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Human Efflux Transporters in Drug Disposition: *in vitro* Transport of Glucuronide Metabolites

Erkka Järvinen

DOCTORAL DISSERTATION

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Abstract

Drug metabolism and transport are key areas in the drug development and therapy. The fate of a drug in the human body is determined by its physicochemical properties, which affect its absorption, distribution, metabolism and elimination processes within the body. The same processes also affect disposition of drug metabolites. Glucuronidation is the most important drug metabolism reaction besides oxidations. Glucuronidation of a drug produces glucuronic acid conjugates that are too hydrophilic to freely permeate cell membranes, and thus they require active transporters for their excretion. Knowledge of disposition of drug metabolites in humans is important for comprehensive understanding of the drug-related effects within the body. For example, glucuronide conjugates of some drugs inhibit drug-metabolizing enzymes and transporters, which causes drug-drug interactions in humans. Therefore, investigations of the molecular mechanisms of drug metabolite excretion are needed.

Hepatic and intestinal ATP-dependent efflux transporters multidrug resistance-associated protein 2 (MRP2, *ABCC2*), MRP3 (*ABCC3*), MRP4 (*ABCC4*) and breast cancer resistance protein (BCRP, *ACBG2*) have been identified to transport glucuronide metabolites of drugs in animal experiments and human in vitro assays. However, systematic studies that compare the properties of these human transporters and characterize their transport kinetics are often lacking. In this thesis, the transport activity of human MRP2-MRP4 and BCRP was evaluated for 18 different glucuronide conjugates of drugs and drug-like compounds, such as androgens and estrogens. The major findings were that MRP2 and MRP3 are rather non-selective transporters and accept most of the glucuronides investigated as their substrates. MRP4 and BCRP, on the other hand, exhibit rather selective transport and these transporters were active only toward some of the glucuronides that were tested in this thesis. P-glycoprotein (P-gp, *ABCB1*), another important drug efflux transporter, was also included in the assays. However, this transporter did not transport any of the glucuronides investigated. Transport kinetic analyses revealed low K_m values for MRP3, mostly clearly below 100 µM. This indicates that MRP3 is a high affinity transporter for glucuronide metabolites in the liver and intestine, where it is highly expressed. The K_m values of MRP2 ranged from 120 to 800 µM. These values suggest that MRP2 is a low affinity, but possibly a high capacity, transporter in the same tissues as MRP3. MRP4 and BCRP exhibited K_m values between 3-170 µM and 10-80 µM, respectively. In conclusion, MRP2, MRP3, MRP4 and BCRP are important efflux transporters that affect disposition of glucuronide metabolites of drugs in the human body. The affinity of glucuronides to these transporters may determine the different excretion of these drug metabolites in vivo, in either urine or bile.

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again amazed me, in addition to bringing the quality of the thesis to the next level, how much fun we have together everyday and are also able to relax besides science.

Helsinki, November 2019

Erkka Järvinen

Contents

List of original publications

This PhD thesis is based on the following original publications:

I Järvinen E, Troberg J, Kidron H, Finel M: Selectivity in the Efflux of Glucuronides by Human Transporters: MRP4 Is Highly Active toward 4-Methylumbelliferone and 1-Naphthol Glucuronides, while MRP3 Exhibits Stereoselective Propranolol Glucuronide Transport. *Molecular Pharmaceutics*, **2017,** 14, 3299-3311.
DOI: 10.1021/acs.molpharmaceut.7b00366

II Järvinen E, Sjöstedt N, Koenderink JB, Kidron H, Finel M: Efflux transport of nicotine, cotinine and *trans*-3′-hydroxycotinine glucuronides by human hepatic transporters. *Basic and Clinical Pharmacology and Toxicology*, **2019**, 125, 490-498.
DOI: 10.1111/bcpt.13281

III Järvinen E, Deng F, Kidron H, Finel M: Efflux transport of estrogen glucuronides by human MRP2, MRP3, MRP4 and BCRP. *Journal of Steroid Biochemistry and Molecular Biology*, **2018,** 178, pp. 99-107.
DOI: 10.1016/j.jsbmb.2017.11.007

IV Järvinen E, Kidron H, Finel M: Human Efflux Transport of Testosterone, Epitestosterone and Other Androgen Glucuronides. *Journal of Steroid Biochemistry and Molecular Biology*, **2019** (In press).
DOI: 10.1016/j.jsbmb.2019.105518

The publications are referred to in the text by their Roman numerals.

Author's contributions

I EJ designed and conducted the transport assays, developed the analytical methods and analyzed samples. EJ had major contribution to the preparation of transporter vesicles and designed the concept of the biosynthesis, while JT did the experimental work of the biosynthesis. EJ analyzed the results and data, and wrote the manuscript with contributions from the co-authors.

II EJ designed and conducted the transport assays, developed the analytical methods and analyzed samples. NS designed and conducted most of the inhibition assays. EJ had major contribution to the preparation of transporter vesicles. EJ analyzed the results and data, and wrote the manuscript with contributions from the co-authors.

III EJ designed and conducted the transport assays with estrogen glucuronides, developed the analytical methods and analyzed samples. FD did most of the estrone sulfate assays. EJ had major contribution to the preparation of transporter vesicles. EJ analyzed the results and data, and wrote the manuscript with contributions from the co-authors.

IV EJ designed and conducted the transport assays, developed the analytical methods and analyzed samples. EJ had major contribution to the preparation of transporter vesicles, designed the concept of the biosynthesis and did the analytical characterizations of the biosynthesis product. EJ analyzed the results and data, and wrote the manuscript with contributions from the co-authors.

Abbreviations

95% CI	95% confidence interval of the data fitting
AADAC	Arylacetamide deacetylase
ABC transporters	ATP-binding cassette transporters
AUC	Area under the curve
BBB	Blood-brain barrier
BCRP	Breast cancer resistance protein
BCHE	Butyrylcholinesterase
CES	Carboxylesterase
CL_{influx}	Transporter-mediated influx clearance
CL_{efflux}	Transporter-mediated efflux clearance
$CL_{efflux,bile}$	Transporter-mediated efflux to bile
CL_h	Hepatic clearance
$CL_{int,h}$	Hepatic intrinsic clearance
CL_{met}	Metabolic clearance
$CL_{passive}$	Passive diffusion clearance
CL_r	Renal clearance
CL_{sec}	Active renal secretion
CL_{sys}	Systemic clearance
CTRL	Control vesicles
$CTRL^{+C}$	Cholesterol supplemented control vesicles
CYP	Cytochrome P450
ECCS	Extended Clearance Classification System
f_a	Fraction absorbed
f_e	Fraction excreted
f_g	Fraction escaping gut-wall extraction
f_u	Fraction unbound in blood
-G	Glucuronide
GFR	Glomerular filtration rate
h	Hill coefficient
K_m	Michaelis constant
MATE	Multidrug and toxin extrusion transporter
MRP	Multidrug resistance-associated protein
MW	Molecular weight
OAT	Organic anion transporter
OATP	Organic anion transporting polypeptide
OCT	Organic cation transporter
PEPT1	Peptide transporter 1
P-gp	P-glycoprotein
PON	Paraoxonase
PSA	Polar surface area
S_{50}	Concentration producing half-maximal transport rate
SLC transporters	Solute carrier transporters
UGT	UDP-glucuronosyltransferase
V_{max}	Maximum transport rate

1 Introduction

Drug development of new small molecular entities requires numerous cycles of optimizations for various biological and molecular properties. Ultimately, efficacy and safety are the most important qualities of drugs, and only when they are acceptable a drug may enter the market and be available for patients. The disposition of a drug in the human body and its pharmacokinetics are among the key factors that control the extent of pharmacological effect of a drug and its off-target effects that might cause toxicity. For example, tissue accumulation of a drug or covalent binding of its reactive metabolites to cell proteins may cause toxic effects, which should be identified and circumvented during the optimization of drug metabolism and transport properties. The disposition and pharmacokinetic profile of a drug is the sum of various drug metabolism and transport processes that occur mainly in the liver but also in other tissues, such as in the intestine and kidney. Thus, the physicochemical and structural properties of a drug do not only determine its potency to a target and off-targets, but also control its permeability across membranes, affinity to a diverse set of different drug-metabolizing enzymes and transporters, and its subsequent elimination from the body (summarized in Figure 1).

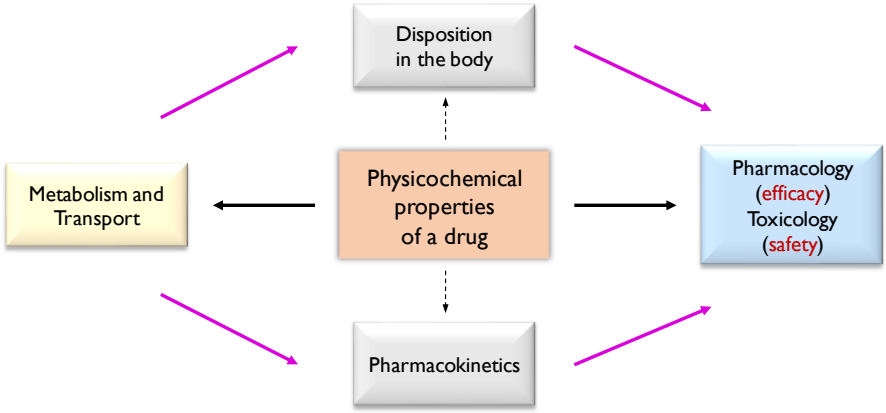


Figure 1 *Important properties of drugs and their relations. Efficacy and safety are the most important endpoints of drug development and they are affected by the exposure of a drug in the human body (pharmacokinetics) as well as its transfer to tissues and elimination from the body (disposition). The physicochemical properties of a drug determine its cellular effects such as permeability and affinity to drug-metabolizing enzymes and transporters, and to targets and off-targets.*

The drug metabolism and transport processes can each be divided into classes of different functions, such as oxidation and conjugation reactions or uptake and efflux transport. Furthermore, these reactions are often studied at the level of individual enzymes and transporters, and depending on the physicochemical properties of a drug and the location of its target in the human body, the right assays must be employed and developed. Although animal experiments are an inevitable part of drug development, the understanding of the precise function of individual human enzymes and cellular processes is required to build up comprehensive knowledge of drug action and its duration in humans. For example, emerging techniques such as physiologically based pharmacokinetic models require incorporation of parameters for the individual processes

that drugs undergo in tissues and cells. Another emerging area is precision medicine, for which the understanding of cellular processes of drugs is needed to translate the effects of genetic variations into clinical pharmacokinetics and disposition of drugs in humans. Lastly, knowledge of molecular processes and mechanisms of drug disposition in humans does not only significantly contribute to drug development but also to better understanding of disposition of endogenous compounds, such as steroids, and toxic byproducts of metabolism, such as bilirubin. Therefore, the findings of this thesis further apply to other small molecular xenobiotics and endogenous compounds, in addition to drugs. The scope of this thesis is to understand the effects of efflux transporters on the disposition of drug metabolites, more specifically glucuronide conjugates.

2 Background

2.1 Drug metabolism and transport

Drug metabolism has traditionally been divided into phase I and II reactions.[1] This concept comprises functionalization and conjugation reactions, which are catalyzed by phase I and II enzymes, respectively. For example, cytochrome P450 (CYP) enzymes typically introduce a hydroxyl group into a drug and subsequently UDP-glucuronosyltransferases (UGTs) attach glucuronic acid to this hydroxyl (Figure 2). However, such classification is partially misleading, because conjugation reactions can be the primary metabolic route of a drug without prior phase I functionalization.[1-3] Additionally, phase 0 and phase III concepts have been introduced to represent transport processes, together with phase I and II, as the whole concept of drug metabolism and transport (Figure 2).[4] Phase 0 denotes the influx transport of a drug into tissues and it consists of passive permeability and carrier-mediated processes that is almost completely accounted for by solute carrier (SLC) transporters. Phase III denotes the active efflux transport of drug metabolites derived from phase II metabolism, which is mostly mediated by ATP-binding cassette (ABC) transporters. Currently, it is accepted that all the phases from 0 to III contribute to the clearance of drugs and affect their disposition in the human body, and that the sequential metabolism from phase I to phase II is not mandatory. Although these concepts help in understanding the different processes in drug metabolism and transport, a comprehensive understanding of all the mechanisms for a drug should be included and addressed as the whole, instead of as individual processes.[5,6]

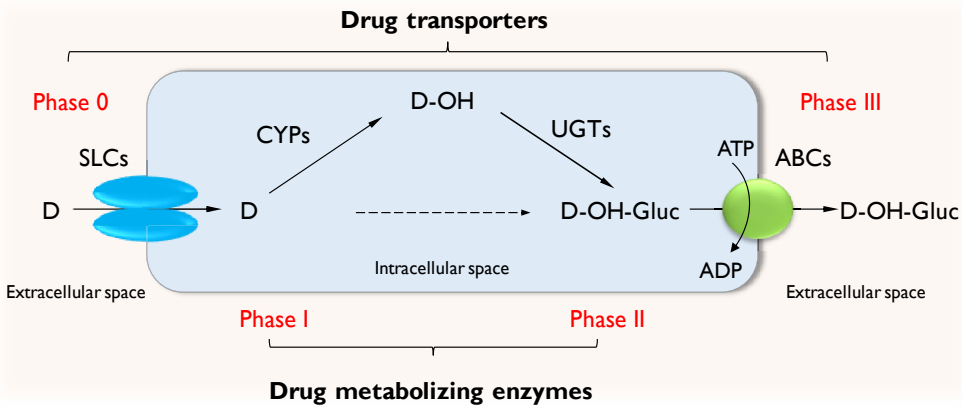


Figure 2 *The general concept of drug metabolism. Phase 0 transporters are part of the SLC family, whereas nearly all phase III transporters belong to the ABC family. A typical phase I hydroxylation reaction is catalyzed by CYP enzymes and it is often followed by conjugation, typically with glucuronic acid (Gluc), which is catalyzed by UGTs. Direct conjugation of a drug is also possible as indicated by the dashed line.*

Drug metabolism reactions and transport processes are present in several tissues including the liver, intestine, kidney and blood.[1,5] The liver is quantitatively the most important tissue in the

clearance of drugs, which is a result of high expression of drug-metabolizing enzymes and transporters as well as the large size of the liver and high blood flow through it. The small intestine is important in restricting the absorption of orally administered drugs to the systemic circulation by different biotransformation reactions and efflux transport back to intestinal lumen.[7] The intestine might also facilitate the absorption of drugs via intestinal uptake transporters, such as the peptide transporter 1 (PEPT1).[7,8] The kidney does not contribute to the first-pass effect of drug metabolism, however, it plays a significant role in the excretion of drugs from the human body to the urine.[5] In addition, biotransformation reactions can occur in the kidney to some extent, which indicates that the kidneys are not only excreting organs.[1]

The main goal of drug metabolism and transport optimization in drug development is the determination of a dose and dose interval for a drug.[5] For a hepatically cleared and orally administered drug, Equation 1 presents the relation between the fraction absorbed (F_a) and the fraction escaping gut-wall extraction (F_g) as well as the hepatic intrinsic clearance ($CL_{int,h}$), the dose interval (τ) and dose. In addition, the pharmacological effect is related to the average plasma concentration ($C_{ss,av}$) that the drug should reach for its effect in the human body.

(1) $$dose = \frac{C_{ss,av} \times CL_{int,h} \times \tau}{F_a \times F_g}$$

Thus, the factors that affect F_a, F_g and $CL_{int,h}$ should be comprehensively known to describe the drug metabolism and transport processes of a drug. F_a is mostly determined by the dissolution and permeation properties of a drug, while F_g is dependent on the metabolic clearances in the intestine.[6,9] $CL_{int,h}$ is the most important factor that affects the total clearance of a drug and it is described and discussed more comprehensively below (Figure 3, Equation 4).

Once a drug reaches the systemic circulation, the systemic clearance (CL_{sys}) is defined not only by the hepatic clearance (CL_h) but also by the clearances in other organs of which the kidneys and, accordingly, the renal clearance (CL_r) are the most important (Equation 2, Figure 3).[10]

(2) $$CL_{sys} = CL_h + CL_r$$

CL_r is the factor of glomerular filtration rate (GFR) and the fraction of unbound drug in blood (f_u) that is summed with the active renal secretion (CL_{sec}) and subtracted by the fraction of drug that is actively and passively reabsorbed (F_{reabs}).[6] The metabolic clearance in the kidney should be included in CL_r if the renal enzymes metabolize a drug. Furthermore, as CL_{sec} is a transporter-mediated process, the value of this term depends on the affinity of a drug to the renal transporters and subsequently it may have high impact on CL_{sys} of some drugs. Equation 3 determines the total renal clearance of a drug.

(3) $$CL_r = f_u \times GFR + CL_{sec} - F_{reabs}(f_u \times GFR + CL_{sec})$$

Finally, the different phases of drug metabolism must be addressed as a whole, as discussed above, and thus the extended clearance model has been introduced for $CL_{int,h}$, as described in Equation 4.[6,10] $CL_{int,h}$ is determined by the active transporter-mediated hepatic influx and efflux clearances over the basolateral membranes of hepatocytes (CL_{influx} and CL_{efflux}), the passive diffusion clearance ($CL_{passive}$), the metabolic clearance (CL_{met}) and the transporter-mediated efflux to bile ($CL_{efflux,bile}$).

(4) $$CL_{int,h} = \frac{(CL_{influx} + CL_{passive}) \times (CL_{met} + CL_{efflux,bile})}{(CL_{efflux} + CL_{passive} + CL_{met} + CL_{efflux,bile})}$$

Equation 4 defines the relations between metabolism and transport and their effect on the hepatic clearance of a drug (Figure 3). First, if a drug is not a substrate, or is a weak substrate, of uptake or efflux transporters and its passive permeability is high in comparison to the metabolic clearance, the metabolism determines alone the overall hepatic clearance ($CL_{int,h} \approx CL_{met}$). Second, if a drug is a low permeable compound and a better substrate for uptake than efflux basolateral membrane transporters, then CL_{influx} will determine the clearance that will be independent of other processes ($CL_{int,h} \approx CL_{influx}$). Third, if the transporter and elimination clearances are in a similar range, it is important to include the contribution of all individual clearances in Equation 4 to the total hepatic intrinsic clearance of a drug. These three examples are simplified scenarios. However, they help understand the different disposition mechanisms and, in particular, they define the vital role of transporters alongside metabolism in the systemic clearance of drugs.

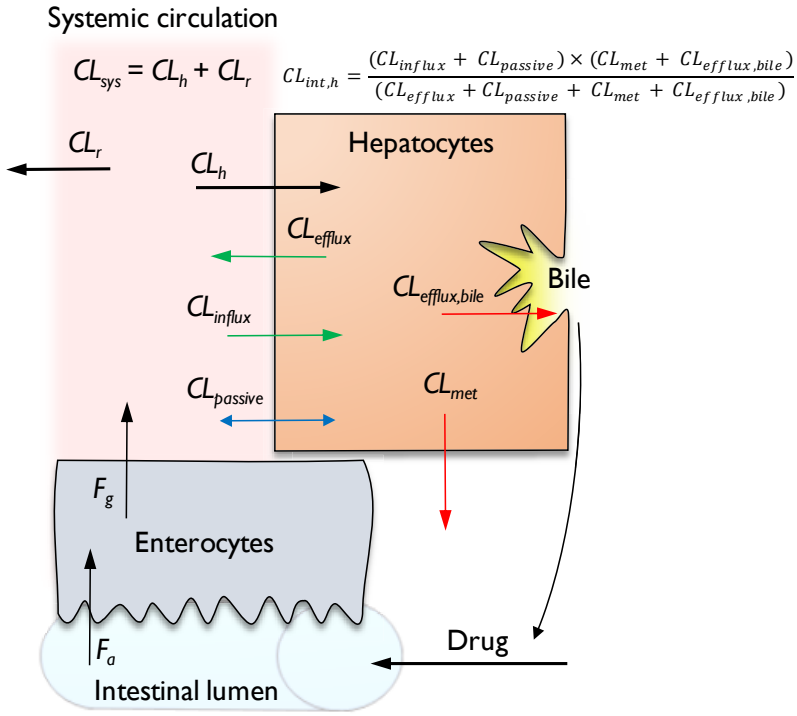


Figure 3 *The main factors that affect the clearance of orally administered drugs. The red arrows are clearances that eliminate a drug, the green arrows are transporter-mediated processes that do not eliminate a drug but affect its systemic clearance and hepatic concentrations. The blue arrow is independent of transporters.*

2.1.1 Overview of drug metabolism reactions

Phase I oxidations are the most important drug metabolism reactions and they are mainly catalyzed by CYP enzymes.[3,11] Up to 85% of the new molecular entities that were approved during

2006-2015 by the United States Food and Drug Administration (FDA) are metabolized via either phase I or II route.[2] CYP-mediated oxidations are the primary metabolic route for 53% of the drugs, whereas of the phase II enzymes UGTs and hydrolases, such as esterases, contribute to the primary metabolism of 12% and 11% of the drugs, respectively. Although UGTs are not the primary clearance route for majority of the drugs, they may contribute to some extent to the metabolism of up to 50% of drugs.[12]

The high impact of CYPs in drug metabolism is due to the various oxidation reactions that they catalyze.[13] CYP-catalyzed reactions utilize molecular oxygen that is activated by heme-iron and the reduced nicotinamide adenine dinucleotide phosphate cofactor.[13] The CYP enzyme reactions usually introduce aromatic or aliphatic hydroxyls into a drug molecule. Hydroxylation of an alkyl group adjacent to an oxygen or nitrogen atom leads to the cleavage of the alkyl chain, which results in O-dealkylation and N-dealkylation reactions, respectively. Hydroxylation and dealkylation reactions are the most important CYP-catalyzed reactions, since they functionalize a drug with a hydroxyl or an amine (Figure 4). These groups are prone to subsequent conjugation reactions that further lead to the excretion of the compound from the human body.[1,4]

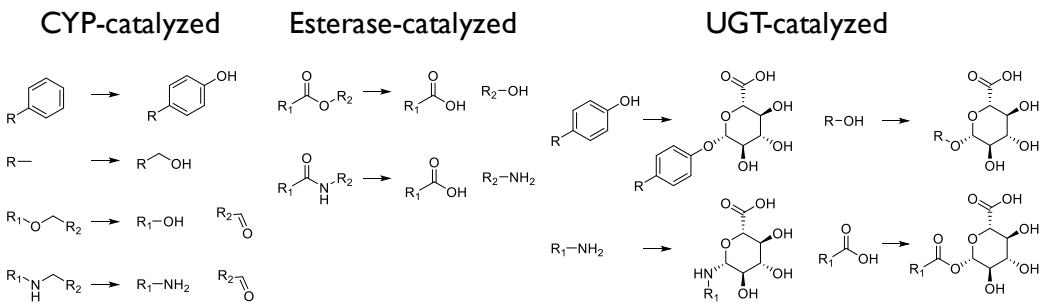


Figure 4 *Typical drug metabolism reactions that are catalyzed by the most important enzyme classes in drug metabolism.*

Of the 57 CYPs, about a dozen are currently considered as the main enzymes in drug metabolism reactions.[14,15] CYP3A4/5 enzymes are distinctly the most important CYPs, since they metabolize 84 of the 142 new drugs that were approved by the FDA during 2013-2017.[16,17] CYP2D6, CYP2C19, CYP2C8 and CYP2C9 are the second most important individual enzymes in the metabolism of the same set of drugs. In particular, CYP2D6, CYP2C9 and CYP2C19 are important enzymes to consider in drug discovery, because these enzymes frequently have polymorphic forms that may remarkably change pharmacokinetics of drugs.[15] Additional CYPs that play a role in drug metabolism are CYP1A2, CYP2A6, CYP2B6 and CYP2E1.[14,15]

Esterases hydrolyze mostly carboxyl ester bonds in drugs but can also act on thioesters, amides and lactones.[18,19] An esterase-catalyzed cleavage of a carboxyl ester bond in a drug molecule releases an alcohol or an amine and a carboxylic acid (Figure 4). Several different esterases are capable of metabolizing drugs but the most important and the best-characterized ones in drug metabolism are carboxylesterase 1 and 2 (CES1 and CES2), whereas others include arylacetamide deacetylase (AADAC), butyrylcholinesterase (BCHE) and paraoxonase 1 and 3 (PON1 and PON3). Moreover, esterases are important enzymes in the liberation of active moieties, such as alcohols, amines or carboxylic acids, of prodrugs.[8] Different hydrolases activate 86% of prodrugs approved by the FDA during 2006-2015.[2] Regardless of whether the hydrolysis leads to either

active or inactive drug metabolite, the reaction produces chemical groups that are prone to subsequent conjugation reactions similar to the ones derived from CYP-catalyzed oxidations.[19] A distinct difference between esterases and other drug-metabolizing enzymes is that some esterases are secreted to the plasma from the liver and can exhibit high activity in blood.[18,19]

UGTs transfer a glucuronic acid from the uridine diphosphate glucuronic acid cofactor to a nucleophilic group in a drug, which results in a glucuronide conjugate of a drug.[19,20] Glucuronidations of aliphatic and particularly aromatic hydroxyls, carboxylic acids and amines are the most prominent glucuronidation reactions (Figure 4). These chemical groups may be derived from CYP-catalyzed oxidations or esterase-catalyzed hydrolyses of a drug, as discussed above. However, many drugs contain within themselves aliphatic and aromatic hydroxyls or carboxylic acids that are readily available for glucuronidation. The UGT enzyme superfamily contains 22 different proteins of which subfamilies 1A and 2B are involved in drug metabolism. UGT1A1, UGT1A3, UGT1A4, UGT1A6, UGT1A9, UGT1A10, UGT2B4, UGT2B7, UGT2B10, UGT2B15 and UGT2B17 are currently known to be important in drug metabolism.[19,21]

Other important drug-metabolizing enzymes include, but are not limited to, aldehyde oxidase (AO), flavin-containing monooxygenases (FMOs), monoamine oxidases (MAOs), sulfotransferases (SULTs) and glutathione transferases (GSTs). CYPs, UGTs and esterases are the main metabolizing enzymes that contribute to the clearance of drugs, although AO, FMOs and MAOs play a role in specific cases and their contribution to drug metabolism might have been generally underestimated.[1-3,11,14,22] FMO-catalyzed oxidations produce nitrogen and sulfur oxides, whereas AO produces mostly lactams and MAOs generate aldehydes, and thus these metabolites are not directly available for phase II conjugation reactions.[22] SULTs, on the other hand, are phase II enzymes that contribute to the conjugation of similar compounds as UGTs, but mostly to a lesser extent.[1,3] GSTs are classified as phase II enzymes and they transfer glutathione to electrophilic carbons, however, this reaction might also happen non-enzymatically in cells.[4,23,24] In addition, glutathione conjugation can be a result of rather complex metabolic processes that involve reactive and possibly toxic metabolites derived from CYP-catalyzed oxidations.[24,25] These reactive metabolites are subsequently deactivated by GSTs or by spontaneous glutathione conjugation.

Human intestinal bacteria are also capable of catalyzing a high number of various biotransformation reactions.[26] Of these reactions, deconjugations are the most prominent ones, particularly because numerous different bacterial β-glucuronidases catalyze the cleavage of glucuronic acid from glucuronide drug metabolites excreted to the intestine via bile.[27,28] In such cases, the deconjugation hampers the elimination of a drug and this unconjugated drug can be reabsorbed from the intestine. The metabolic activity of bacteria may differ between the intestinal segments, and, for example, β-glucuronidase activity increases highly toward the colon.[28] Nevertheless, orally administered drugs are exposed to the intestinal bacteria and thus the contribution of bacteria to the metabolism of drugs should not be overlooked.

2.1.2 Drug metabolism in the intestine, liver and kidney

Most drugs are administered orally, which means that a drug must pass the intestinal epithelium before it reaches the portal vein and subsequently the liver. However, the intestinal barrier restricts the absorption of drugs and other exogenous compounds in humans not only physically but also by expressing several drug-metabolizing enzymes in enterocytes. The first-pass clearance of a drug must be assessed as two separate clearance processes, one in the intestine and the other one

in the liver.[5] These two factors, F_g and the fraction escaping hepatic extraction (F_h), can differ markedly for the same drug, which emphasizes the contribution of different enzymes in both the liver and intestine.[9,29] This difference is particularly true for UGTs whose enzyme expression patterns vary substantially between the liver and intestine (Table 1), which might contribute to the low F_g of drugs that are substrates for UGTs, as discussed below.[30,31]

Table 1. *The main drug-metabolizing enzymes in the intestine, liver and kidney. See the text for details and references.*

Enzyme	Intestine	Liver	Kidney
CYP	CYP3A4	CYP3A4	CYP2B6
	CYP2C9	CYP2C9	CYP3A5
		CYP1A2, CYP2A6, CYP2C8	
		CYP2B6, CYP2D6, CYP2C19	
		CYP3A5	
UGT	UGT2B17	UGT1A1, UGT2B7	UGT1A9
	UGT1A1	UGT1A4, UGT1A9, UGT2B4, UGT2B10, UGT2B15	UGT2B7
	UGT1A10	UGT2B17, UGT1A3, UGT1A6	UGT1A6
	UGT2B7		
Esterase	CES2	CES1	CES2
	AADAC	AADAC	
		PON1, PON3, BCHE	
		CES2	

The intestine expresses drug-metabolizing enzymes along its whole length from duodenum to rectum, although the absolute amounts and expression pattern of the enzymes vary with different segments, and the most enzymes are expressed only in the small intestine.[32-36] Drug absorption occurs mainly in the small intestine and especially in its proximal parts, which also have the highest expression of the drug-metabolizing enzymes.[34,37]

CYP3A4 is quantitatively the most abundant CYP enzyme in the human small intestine and it constitutes over 80% of the drug-metabolizing CYPs in this tissue.[34,38,39] The second most abundant CYP enzyme in the small intestine is CYP2C9, which represents 10-20% of the drug-metabolizing CYPs, whereas CYP2C19, CYP2D6 and CYP3A5 constitute a few percent of the intestinal CYP enzymes. The absolute protein concentration of CYP3A4 in the small intestine is equal to the liver. This similarity is also reflected by the activity of CYP3A4 that is similar in microsomal preparations from the human small intestine and liver.[40] On the other hand, the activities of CYP2C9, CYP2C19 and CYP2D6 are present in intestinal microsomes but only to some extent.[41] F_g value for drugs that are substrates for CYP3A4/5 can be as low as 0.1, whereas $F_g > 0.75$ is observed for drugs that are metabolized by CYP2C9, CYP2C19 and CYP2D6, which emphasizes the major contribution of CYP3A4 to drug metabolism in the human small intestine.[42,43]

The drug-metabolizing UGTs are expressed either in both the liver and extrahepatic tissues or only extrahepatically.[19,20] The most prominent feature among UGTs is UGT1A10, which is not expressed in the liver but it exhibits high expression along the intestine.[30-32,44] Other UGTs expressed in the intestine are UGT1A1, UGT2B7 and particularly UGT2B17 that has the highest expression level of the intestinal UGTs in humans. Nakamori and coworkers demonstrated an inverse correlation between the clearance in human intestinal microsomes and $F_a \times F_g$ for 11 drugs that are substrates for human UGTs.[45] The lowest calculated $F_a \times F_g$ values were for raloxifene (0.05) and quercetin (0.002), and these values were much lower than has been reported for drugs

that are substrates of CYP enzymes.[42,45] More importantly, Cubitt and coworkers demonstrated six- and nine-fold higher scaled clearance values for raloxifene and troglitatzone in the human intestine than liver.[46] The intestine-specific UGT1A10 catalyzes the glucuronidation of raloxifene and troglitatzone, which explains the high clearance of both drugs in the small intestine and the low F_a x F_g value for raloxifene.[47,48] On the other hand, the activity of UGT1A10 only partly explains the high intestinal glucuronidation of quercetin but the activity of UGT1A1 might play a higher role in this case.[45] The activity of UGT2B7 in the intestine has been demonstrated to restrict oral bioavailability of morphine but to a much lesser extent than drugs that are highly glucuronidated by UGT1A10.[49,50] The high expression of UGT2B17 in the human intestine contributes to the low bioavailability of testosterone.[36,51] Together, four UGTs highly catalyze glucuronidation of drugs in the small intestine and restrict their absorption, while of the CYPs only CYP3A4 is highly expressed and active in the intestine.

The most important human intestinal esterase is CES2 that is also expressed in the liver, whereas CES1 is absent from the intestine.[52] In the liver, on the other hand, the expression of CES1 is about 20- to 30-fold higher than the expression of CES2.[53] AADAC is expressed similarly in both tissues, while PON1, PON3 and BCHE are expressed highly only in the liver but are also secreted to the plasma, and together they contribute to the inactivation of several drugs.[18,19,54] Although the substrate preferences of CES1 and CES2 overlap, the absolute hydrolysis rates can differ between these two enzymes. For example, CES2 hydrolyses the prodrug prasugel in the intestine to a metabolite that is further converted by CYP3A4 to an active metabolite.[55] In addition, CES1 hydrolyses prasugel in the liver, while AADAC contributes to approximately half of the hydrolysis in both the intestine and liver, indicating overlapping substrate specificity between all three of these esterases.[19,55] Clopidogrel, on the other hand, is hydrolyzed in the liver only by CES1 to an inactive metabolite and this hydrolysis reaction is undetectable in intestinal microsomes.[56] Furthermore, the human plasma PON1 hydrolyses 2-oxo-clopidogrel, a CYP3A4-derived metabolite of clopidogrel, to an active thiol metabolite, while CES1 inactivates these two metabolites of clopidogrel, as well as the parent drug itself.[57] Together, the aforementioned esterases have a high impact on drug metabolism in the intestine, liver and plasma.

In the liver, CYP3A4 is also the most prominent drug-metabolizing enzyme, but this tissue expresses several other CYPs that are absent from the small intestine.[34,58] CYP2C9 is the second most highly expressed enzyme in the liver, and its expression is almost the same as the expression of CYP3A4. The expression of CYP1A2 is half in comparison to CYP3A4, while CYP2A6, CYP2B6, CYP2C8, CYP2C19, CYP2A6 and CYP3A5 are expressed at levels of 10 to 30% of the expression of CYP3A4. Although the average hepatic protein expression of CYP3A5 is lower than for CYP3A4, its role in drug metabolism might be much greater, because the *CYP3A5* gene is highly polymorphic leading to great variation in its protein expression.[34,58,59] In addition, CYP3A4 and CYP3A5 are highly homologous enzymes, exhibiting similar clearances for the same compounds.[59] In fact, CYP3A5 might have an even higher or similar contribution as CYP3A4 to the clearance of some drugs in individuals who express a high amount of CYP3A5 protein in their livers.

The liver expresses several UGTs, including some that are absent from the intestine.[30,31] UGT1A1 and UGT2B7 are the main UGTs in the human liver. Other highly expressed hepatic UGTs are UGT1A4, UGT1A9, UGT2B4, UGT2B15, while the expression of UGT1A3, UGT1A6, UGT2B10 and UGT2B17 is a few-fold lower. UGT1A4 and UGT2B10 are particularly significant hepatic enzymes, since they catalyze glucuronidation of amines, whereas the other UGTs are rarely able to catalyze that activity.[19,60] Thus, these two UGTs have an essential role in the clearance of amine containing drugs. UGT1A1 is another highly important UGT, because it

solely catalyzes the detoxification of the endogenous heme metabolite bilirubin.[19,20] Other hepatic UGTs usually overlap in their substrate preferences, and thus all these enzymes are involved in drug metabolism reactions to some extent.[19,21]

The UGTs expressed in the human kidney are UGT1A6, UGT1A9 and UGT2B7.[30,31] The expression levels of UGT1A6 and UGT2B7 are lower in the kidney than in the liver, whereas UGT1A9 is expressed at a three-fold higher level in the kidney than in the liver. Although the kidney does not contribute to the bioavailability of drugs, it might affect the total systemic clearance of some drugs, as discussed above in Section 2.1. However, blood flow through kidneys and the tissue size are smaller than the respective values for the liver.[61] Gill and coworkers measured higher clearance for the UGT1A9 substrates mycophenolic acid and propofol in microsomes from the kidney than liver, while for UGT2B7 substrates the clearance was similar in both preparations.[61] The renal UGT1A9 appears to contribute significantly to the systemic metabolism of propofol, because the metabolic renal clearance of this drug in the human kidneys is almost equal to its hepatic clearance.[62]

CYP2B6 and CYP3A5 are expressed in the human kidneys, while the expression of CYP3A4 is undetectable or low.[38,63,64] Interestingly, in addition to glucuronidation, propofol is metabolized by CYP2B6, which might contribute to the renal metabolism of this drug.[65] Low expression of CYPs implicates that the kidney has a low contribution to CYP-catalyzed drug metabolism and drug-drug interactions, whereas renal glucuronidation clearly has an impact on the systemic clearance of some drugs, and should be considered in specific cases.[61,64] CES2 is expressed in the human kidney while CES1 is absent, which is similar to the expression pattern of these esterases in the small intestine.[52] In addition, AADAC is not expressed in the kidney.[54] No other esterases involved in drug metabolism are expressed in the kidney or are currently identified in this tissue.[18,19]

2.1.3 Overview of drug transporters

The two types of transporters involved in drug disposition are uptake and efflux transporters. Uptake transporters concentrate their substrates from the extracellular space into the cells, whereas efflux transporters carry their substrates from the inside of the cells to the extracellular space over the membrane (Figure 2). Uptake transporters belong to the SLC family, whereas most of the efflux transporters are part of the ABC family, members of which utilize energy from ATP hydrolysis to drive their transport.[66,67] Contrary to ABC transporters, SLC transporters do not hydrolyze ATP but, depending on the transporter, might exchange or cotransport their substrates with sodium ions, protons or other ionic species.[66,68] Together the ABC and SLC superfamilies contain several hundred different transporters of which only a small subset is currently known to be important in drug disposition.[7,66,67,69] Drug transporters are expressed in the same tissues as drug-metabolizing enzymes, namely in the liver, intestine and kidney (Table 2).[7] In contrast to drug-metabolizing enzymes, drug transporters play a pivotal role in the blood-brain barrier (BBB) by restricting the access of drugs to the brain, and thus transporters significantly affect drug disposition and efficacy also in the human central nervous system.[7,70] In addition, efflux transporters protect many tissues from drugs, such as at the placenta by restricting the access of drugs and other foreign compounds to the fetus.[71]

Currently, the most important uptake transporters in drug disposition are organic anion transporting polypetides 1B1, 1B3 and 2B1 (OATP1B1, OATP1B3, OATP2B1) and organic anion transporters 1-3 (OAT1, OAT2, OAT3), which are involved in the hepatic and renal uptake

transport of anionic and zwitterionic drugs and their metabolites.[7,69,72,73] Furthermore, organic cation transporters 1 and 2 (OCT1 and OCT2) are important for the uptake of cationic drugs into the liver and kidney, respectively.[7,69,73] Lastly, other uptake transporters of emerging interest include OAT7, OATP4C1, PEPT1, the sodium/taurocholate co-transporting polypeptide, heteromeric organic solute transporter and the equilibrative nucleoside transporters 1 and 2.[7,8,69,73,74]

Table 2. *The main drug transporters in the intestine, liver and kidney. The gene names are indicated in brackets. See the text for details and references.*

	Intestine	Liver	Kidney
Uptake transporters	PEPT1 (*SLC15A1*)	OATP1B1 (*SLCO1B1*) OATP1B3 (*SLCO1B3*) OATP2B1 (*SLCO2B1*) OAT2 (*SLC22A7*) OCT1 (*SLC22A1*)	OAT1 (*SLC22A6*) OAT3 (*SLC22A8*) OCT2 (*SLC22A2*)
Efflux transporters	P-gp (*ABCB1*) BCRP (*ABCG2*) MRP2 (*ABCC2*) MRP3 (*ABCC3*) MRP4 (*ABCC4*)	P-gp (*ABCB1*) BCRP (*ABCG2*) MRP2 (*ABCC2*) MRP3 (*ABCC3*) MRP4 (*ABCC4*) MATE1 (*SLC47A1*)	MATE1 (*SLC47A1*) P-gp (*ABCB1*) MRP2 (*ABCC2*) MRP4 (*ABCC4*)

Breast cancer resistance protein (BCRP) and P-glycoprotein (P-gp, MDR1) are unequivocally the most important efflux transporters that affect the drug disposition and absorption in several different tissues, including the intestine, liver and kidney.[7,69] The clinical significance of other efflux transporters has not been proven as comprehensively as for the uptake transporters. Thus, besides BCRP and P-gp, only multidrug and toxin extrusion transporters 1 and 2K (MATE1 and MATE2K) are currently known to be involved in drug-drug interactions or have polymorphic variations that affect the pharmacokinetics and efficacy of drugs.[69] Multidrug resistance-associated proteins 2, 3 and 4 (MRP2, MRP3 and MRP4) still lack robust clinical evidence of their contribution to drug-drug interactions or polymorphic variations in the pharmacokinetics of drugs.[7,69] However, overall evidence suggests that they are the main carriers for anionic drugs and drug metabolites in the intestine, liver and kidney and, therefore, are clinically highly relevant transporters.[7,69,73,75]

Identification of the contribution of specific transporters to drug disposition is challenging, because the tissue uptake and efflux of a drug is not always quantifiable in vivo in humans and does not result in the biotransformation of a drug, such as in the case of drug metabolism reactions.[7,75,76] In addition, deriving influx and efflux parameters from the net transport into the tissue, such as in the liver, might be impossible. Hence, it is obvious that the landscape of drug transporters is not yet established as completely as for drug-metabolizing enzymes, and the knowledge of transporters will expand in the future.

2.1.4 Drug transporters in different tissues

The small intestine exhibits high expression levels of efflux transporters in enterocytes, both in the apical membranes facing the intestinal lumen and in the basolateral membranes at the blood circulation side (Figure 5). BCRP and P-gp are localized to the apical side and exhibit increasing

expression levels toward the ileum, while their levels in the colon are lowest along the intestine.[7,77,78] MRP2 and MRP3 are efflux carriers in the intestine with higher expression levels than BCRP and P-gp, particularly in the colon where they are the two main transporters. MRP2 is localized to the luminal side of enterocytes, while MRP3 is localized to the basolateral membranes. MRP4 is another intestinal transporter from the MRP family, which is expressed at similar levels as MRP3 along the whole intestine, but the localization of MRP4 has not been confirmed yet in human intestinal tissues.[77] MRP4 is localized to the apical membranes of cells of the human epithelial colorectal adenocarcinoma cell line, Caco-2.[79] Additional evidence for the basolateral localization of MRP4 in enterocytes comes from a knockout mouse study.[80] That study found lower basolateral transport of the MRP4 substrate cephadroxil across isolated jejunal tissue from mice lacking Mrp4 compared to the tissue from wild type mice.

The small intestine expresses a few uptake transporters in the apical membranes of enterocytes and these transporters might facilitate the drug absorption.[7,69,73] PEPT1 is essential for the absorption of small peptides but it also carries drugs, and thus it plays a role in the absorption of peptide-like drugs or prodrugs designed to be carried by this transporter.[8,69,78] OATP-mediated absorption of some hydrophilic drugs has been postulated and OATP2B1, a rather highly expressed transporter in the intestine, might be responsible for these observations.[77,78,81,82] However, the localization of OATP2B1 in enterocytes is under debate and future studies are warranted to clarify whether this transporter affects drug disposition, drug absorption or both in enterocytes.[73,82]

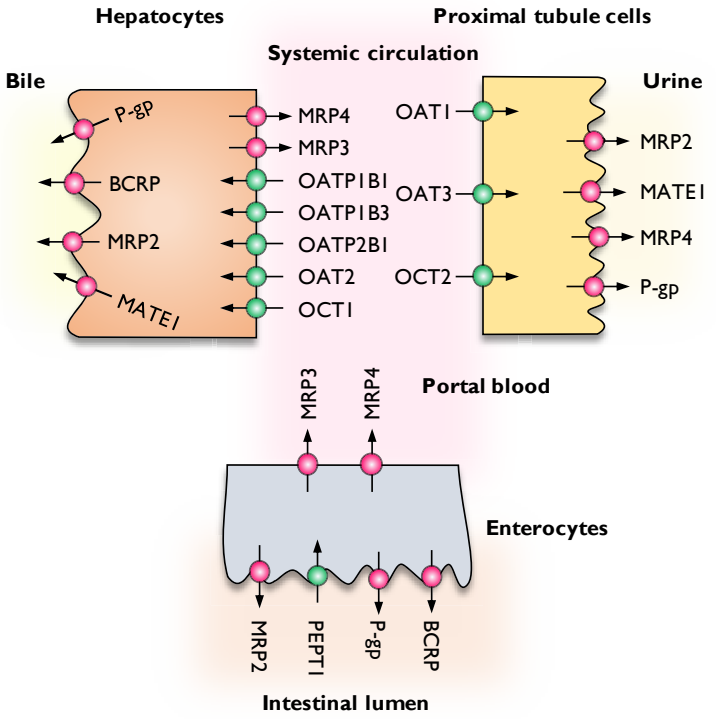


Figure 5 *Localization of the main drug transporters in hepatocytes, enterocytes and proximal tubule cells. In addition to the indicated transporters, multiple other drug carriers are expressed in these tissues. See text for details and references.*

The liver expresses almost the same main efflux transporters as the intestine, but their relative expression levels are different.[77,83] The expression of BCRP is low in the liver, whereas the abundance of P-gp is clearly higher but its amount is only half of its intestinal amount. The expression of MRP2 is higher in the liver than along the intestine, whereas the expression of MRP3 is similar to the intestine, and MRP4 is almost undetectable in the liver. Localization of the aforementioned transporters is similar in hepatocytes as in enterocytes that is BCRP, P-gp and MRP2 are found in the apical membranes at the bile side, while MRP3 is localized to the basolateral membranes at the blood side (Figure 5).[7] Furthermore, MRP4 is localized to the basolateral membranes of hepatocytes.

The liver also expresses several other efflux transporters, which are presumably associated with drug disposition or toxicities. The highly expressed apical membrane transporters are bile-salt export pump and multidrug resistance protein 3, which are vital to maintain bile homeostasis by transporting bile acids and phosphatidylcholine, respectively.[7,69,73,77,83] These transporters are not known to affect drug disposition, but their inhibition by drugs or drug metabolites might lead to impaired formation of bile and potentially to drug-induced liver injury.[69,73,84] MATE1 is a canalicular transporter in hepatocytes and it exhibits similar protein expression as MRP2 and P-gp.[69,77,83] Although MATE1 affects disposition of some drugs in the liver, this transporter might play a more prominent role the in the renal secretion than biliary excretion of drugs. Other MRPs localized to the basolateral membranes of hepatocytes are MRP1, MRP5 and MRP6.[85] Some studies have reported similar protein expression of these transporters as MRP2 and MRP3, although the roles of MRP1, MRP5 and MRP6 in drug disposition are not well-established.[83,85]

Several uptake transporters are highly expressed in the liver and affect disposition of many drugs.[7,68,69] OATP1B1 and OATP1B3 are highly expressed in the basolateral membranes of hepatocytes and are the most comprehensively studied transporters, which carry drugs into hepatocytes from the systemic circulation (Figure 5).[68,77,83] OATP2B1, OAT2, and OCT1 are expressed only at a few-fold lower protein levels than OATP1B1 and OATP1B3 in the same membrane.[77,83] The protein expression of these uptake transporters is higher in comparison to the hepatic efflux transporters MRP2-4, BCRP and P-gp.

In the kidney, the transporters are expressed both in the apical and basolateral membranes of proximal tubule cells where they may contribute to the active renal secretion or reabsorption of drugs.[7,69] MRP4 is localized to the apical membranes of proximal tubule cells alongside MRP2, P-gp and MATE1, and these proteins contribute to the active secretion of their substrates in urine. The protein expression levels of MRP2, MRP4 and P-gp are similar, whereas the expression of MATE1 is a few-fold higher.[86-88] OAT1, OAT3 and OCT2 are the most highly expressed renal uptake transporters in the basolateral membranes of proximal tubule cells and they contribute to the active excretion of drugs.[7,86,88] While OAT2 is also localized to the basolateral membranes of these cells, its expression level is several-fold lower than the levels of OAT1 and OAT3, and thus its effect on the active renal secretion of drugs is not yet established.[73,86,88] The renal expression of BCRP, MRP3 and MATE-2K is low or undetectable.[86-88] Therefore, their role in the renal secretion of drugs may be negligible.

The BBB is perhaps the most important tissue for drug disposition apart from the liver, intestine and kidney. Although several transporters are expressed in the epithelial cells of the BBB, BCRP and P-gp are the most important efflux transporters restricting the brain availability of drugs.[7,70,89] Evidence from numerous reports has implied that BCRP and P-gp play a synergistic role in restricting the distribution of drugs into the brain from the systemic circulation.[70] The efficacy of these two transporters almost completely abolishes access of multiple drugs into brain.

2.2 Excretion of drugs and disposition of their metabolites

A drug is cleared from the body by direct excretion in urine or bile, or after conversion to metabolites by drug metabolism reactions.[5] However, the excretion of a drug is complete only after all drug-related material including metabolites exit via urine or feces from the human body. Drug metabolites may possess similar, or even higher, pharmacological activities compared to the parent drug or be the cause for toxic effects of drugs.[90] Therefore, understanding the disposition of drug metabolites, not only of the parent drug, is an inevitable part of the development of new drugs.[90,91]

2.2.1 Effects of transporters and physicochemical properties on the excretion routes

The main excretion routes for drugs and drug metabolites are via urine or feces.[5] Urinary excretion is driven by glomerular filtration of an unbound compound in blood and by the net effect of active secretion and reabsorption as well as passive reabsorption in the kidney (Equation 3). Biliary excretion is the first step in the excretion of drugs in feces, although in some specific cases enterocytes can directly eliminate drugs from the systemic circulation, which can result in a phenomenon called enteroenteric circulation.[92,93] Examples of enteroenteric circulation in humans are rare, albeit this excretion route might be important for some drugs; for example, the intestinal secretion contributes to one-third of the total excretion of urate, an endogenous compound, in mice.[94] On the other hand, biliary excretion of unchanged drugs is rather common.[28] In most cases, it leads to enterohepatic circulation of drugs and this drastically prolongs their half-life. A representative example of a drug that undergoes enterohepatic circulation is atovaquone.[95] This drug exhibits negligible metabolism and excretion in urine but has high biliary excretion, which prolongs its half-life to several days in humans. Such a long half-life indicates that the drug is extensively reabsorbed from the small intestine and only slowly excreted in feces. Hence, biliary excretion should be considered as a distribution rather than an excretion mechanism, even in the case of some drug metabolites such as glucuronides, which may be efficiently deconjugated in the intestine, leading to reabsorption of the parent drug.[27,28]

It is well established that physicochemical properties determine the extent of metabolism and transport of drugs and their metabolites.[5,6,9,96,97] Nevertheless, predicting the fate of an individual drug is challenging even though some general correlations exist between molecular properties and in vivo outcome. Drugs and drug metabolites excreted in urine are hydrophilic and often carry a charge, because lipophilicity increases passive reabsorption in the kidney and prevents the excretion of drugs in urine.[5,98] In addition, CL_r is decreased proportionally with f_u, whereas lipophilicity further decreases the f_u value.[5,96] Similarly, biliary excretion is also limited for drugs that possess high passive permeability.[92,99] Highly lipophilic drugs distribute back to blood from the hepatocytes and therefore are less prone to be excreted and concentrated in bile. Furthermore, a drug must permeate the hepatic basolateral membrane before excretion in bile, and thus the uptake into the liver can be the rate-liming step prior to efflux transporter-mediated biliary excretion of the drug.[10]

In addition to lipophilicity, the MW and ionization state of a drug affect its biliary excretion.[28,92,100] In rats, anionic drugs are more prone to biliary excretion than cationic, zwitterionic or neutral species.[99] Furthermore, a MW threshold of about 400 Da for biliary excreted anions has been determined in rats, whereas the corresponding value for humans was

reported to be about 500 Da.[99-101] Interestingly, the physicochemical property space of biliary excreted drugs overlaps with the substrates of rat Oatp1b2 and three human hepatic OATPs, and these drugs are mostly anions with a mean MW of about 500 Da.[99] In addition to the MW and negative charge, higher polar surface area (PSA) of drugs correlates with the extent of biliary excretion in rats but also with the substrates of human hepatic OATPs, which have a mean PSA value of 126 Å^2.[99,102] These observations strongly indicate that the hepatic basolateral organic anion uptake transporters, together with the organic anion efflux transporters in the canalicular membranes, play a key role in the biliary excretion of anionic drugs and drug metabolites. Thus, the excretion in urine and bile are the main elimination routes for low permeable compounds, whereas lipophilic drugs have to undergo metabolism before excretion as drug metabolites with reduced lipophilicity.[5]

Several different classification systems are available for the prediction of disposition mechanisms of drugs based on their physicochemical properties, the rate of metabolism and permeability.[103] The extended clearance classification system (ECCS) classifies drugs into four main classes: high permeable acids and zwitterions (Class 1), high permeable bases and neutral drugs (Class 2), low permeable acids and zwitterions (Class 3), low permeable bases and neutral drugs (Class 4).[6] Acids and zwitterions are further separated by MW with a cut-off value of 400 Da. Thus, acidic and zwitterionic drugs below 400 Da are classified in Class 1A and 3A, whereas larger compounds are in Class 1B and 3B.

The ECCS improves the understanding of the effect of physicochemical properties on the clearance mechanisms of drugs and the role of transporters. Class 1A and 2 compounds are mostly cleared by metabolic reactions, whereas renal clearance is observed only for low permeable drugs in Class 3 and 4. Hepatic uptake transporters mostly affect the disposition of compounds in Class 1B and 3B, whereas renal transporters might affect the clearance of compounds in Class 3 and 4. It should be noted that although the hepatic uptake would be the clearance mechanism for most of Class 1B drugs, these drugs are anticipated to be highly metabolized in the liver and the uptake transport is only the rate-liming step for the clearance. Effects of efflux transporters on drugs in different ECCS classes are less robustly determined, although their contribution would be most important for the low permeable compounds in Class 3 and 4.[104] The intestinal absorption process is predicted to be complete (F_a~1) for drugs in Class 1 and 2, whereas drugs in Class 3 and 4 may have lower intestinal absorption that is further decreased by efflux transporters.

The basis for the MW cut-off value in ECCS classes 1A and 3A is the preference of OATPs to transport higher MW anions, as discussed above.[99] This preference correlates with the MW threshold, 400-500 Da, of the majority of biliary excreted anionic compounds in humans and rats.[28,99-102] On the other hand, renal excretion is negligible for highly passive permeable compounds, as discussed above, and therefore only compounds in ECCS Class 3 and 4 are mainly excreted as such in urine.[5,98,99] Because of the complex relationship between the uptake transport, efflux transport and metabolism, the prediction of biliary excretion is challenging. Therefore, the biliary excretion of the parent drug is presumably the predominant clearance route only for Class 3B compounds, which are substrates for hepatic OATPs but do not undergo metabolism. Nevertheless, biliary excretion of other low passive permeable drugs in Class 3A and 4 may also be possible.

Recently, the hepatic OAT2 was identified to transport several ECCS Class 1A drugs, which are not carried by OATP1B1.[72] Furthermore, the renal OAT1 and OAT3 carry several drugs from Class 3A and 4, and some drugs from Class 1A.[105] Thus, the total systemic clearance of drugs is a complex effect of different transporters, including OATs and OATPs, which affect the hepatic and renal clearance. The current understanding of intrinsic molecular properties of drugs that

affect the elimination route and effects of transporters on the elimination of drugs are summarized in Table 3.

Table 3. *The main elimination routes for drugs, the transporter effects and the intrinsic molecular properties of drugs that affect their elimination. ECCS classification is indicated.*[5,6,72,98,100,101,104,105]

Main elimination route	Effect of the intrinsic molecular properties and transporters, and ECCS classification
Metabolism	logD > 0, PSA < 75 Å², high permeability, F_a~1 ECCS 1A, 1B and 2 Transporters: Hepatic OAT2 (ECCS 1A) and OATPs (ECCS 1B) as the rate-limiting step in the elimination
Biliary	MW > 400-500 Da, PSA > 75 Å², a negative charge, low to medium permeability and LogD ECCS 3B Transporters: Hepatic OATPs, intestinal and hepatic efflux transporters (P-gp, BCRP, MRP2)
Renal	logD < 0, PSA > 75 Å², high f_u, low permeability ECCS 3A, 3B and 4 Transporters: Renal OAT1, OAT3 and OCT2, intestinal efflux transporters

2.2.2 Disposition of drug metabolites

Prediction of the systemic exposure is important for the evaluation of safety and activity of drug metabolites.[106] The systemic exposure also affects the renal clearance of a drug metabolite, since only the unbound compound in blood can be excreted in urine.[5] Thus, it is important to understand the mechanisms that determine the excretion routes and the extent of systemic exposure of drug metabolites, not only the parent drug. Most of the pharmacologically active metabolites are derived from oxidative and reductive metabolic reactions that result in minor modifications to a drug, such as hydroxylation. In addition, hydrolysis of prodrugs usually releases an active carboxylic acid metabolite.[3,8,90] Apart from prodrugs, conjugation or hydrolysis reactions do not typically produce active or toxic metabolites, although several examples are found among them that are active or toxic.[3,90] For example, the glucuronide conjugates of the cholesterol absorption inhibitors S-8921 and ezetimibe are 6000- and several-fold more potent inhibitors than their parent drugs, respectively.[107,108] In addition, some glucuronide conjugates mediate drug-drug interactions. For example, clopidogrel and gemfibrozil glucuronides inhibit both CYP2C8 and OATP1B1.[109] Furthermore, glucuronide metabolites of several nonsteroidal anti-inflammatory drugs were found to inhibit the renal uptake and efflux transporters OAT1, OAT3, MRP2 and MRP4, although the clinical significance of the inhibition is not yet known.[110] Hence, more interest has recently been raised in glucuronide metabolites.

Oxidative metabolic reactions, such as hydroxylation or dealkylation, generally result in a reduction of no more than a couple of logP units in lipophilicity of the metabolite compared to the parent drug.[111-113] Such a small decrease means that the metabolites derived from oxidation reactions usually retain similar permeation properties as the parent drug, if the drug itself is highly permeable. In the aforementioned cases, the systemic exposure of metabolites will be determined by the fraction metabolized via this specific route.[111,114] If the specific metabolic route is

dominant, the exposure of the metabolite is often high. However, some metabolites might exhibit negligible systemic exposure even though the major clearance of the drug is via this specific route and the formed metabolite is also found in human excreta.[111,115] In particular, multistep metabolic reactions, such as aromatic oxidation, which results in a phenol that is subsequently conjugated with glucuronic acid or sulfate, leads to a high systemic exposure of the conjugate but to low systemic exposure of the first formed metabolite. In vitro the opposite might be true. More discrepancies between the fraction metabolized value and the systemic exposure are observed for hydrophilic metabolites, whose disposition is restricted by membrane-permeability and depends on active transporters.[111,113,115] For example, conjugated metabolites and particularly glucuronides might reach high systemic exposures.[113] In some cases, glucuronides may have drastically different disposition in humans than what is anticipated based on the in vitro fraction metabolized by glucuronidation of the parent drug. For instance, glucuronidation is the only metabolic route for cabotegravir and pradigastat in vitro, but their glucuronide conjugates were not found in the systemic circulation in humans.[116,117] Pradigastat is completely excreted in feces, whereas cabotegravir is partly excreted in urine as cabotegravir-glucuronide but most of the dose is excreted as the parent drug in feces. In both cases, the glucuronide metabolite is most likely excreted via bile to the intestine and then deconjugated by bacterial β-glucuronidase that releases the parent drug.

Glucuronidation, the most important conjugation reaction, results in a reduction of lipophilicity of a drug by several logP units and adds a negative charge to it.[113,118] Furthermore, glucuronidation increases the MW of a drug by 176 Da, which mostly leads to metabolites with MWs above 400 Da since the MW of a typical drug is 200-600 Da.[96] Glucuronide metabolites demonstrate over an order of magnitude, typically 50-fold, decrease in the permeability compared to their parent drugs.[119-124] Such a decrease in the permeability is distinct in comparison to metabolites originating from oxidative metabolism. Therefore, the human exposure of glucuronide metabolites is not fully predictable only based on the knowledge of the extent of glucuronidation. This is because the membrane-barrier restricts the diffusion of glucuronides, and thus the permeation is affected by the efficiency of different transporters rather than by the physicochemical properties of the metabolite alone.[113,125]

Carboxylic acid metabolites, derived from oxidation or hydrolysis reactions, might exhibit permeability-limited systemic exposure and therefore transporters may affect their systemic disposition and excretion in either bile or urine.[111] For example, losartan is metabolized to a carboxylic acid metabolite, which has a 20-fold lower passive permeability but only 2-fold lower total influx, including passive and active, than the parent drug in human hepatocytes.[126] Furthermore, the carboxylic acid metabolite of losartan has a 50-fold higher basolateral efflux than losartan, which agrees with several times higher systemic concentration of the metabolite in comparison to the parent drug in humans. Such an example elegantly emphasizes the importance of active basolateral carriers in determining the systemic exposure of carboxylic acid metabolites. Similarly, membrane-limited permeability controls the disposition of the dicarboxylic acid metabolite enalaprilat, which is formed from the monocarboxylic acid parent drug enalapril.[127] Enalaprilat reaches seven-fold higher systemic exposure than enalapril in humans. In addition, 60% of the total excreted enalaprilat is found in urine and the rest in feces, which indicates that both apical and basolateral transporters are similarly active in the human liver. In perfused rat livers, the basolateral efflux of enalaprilat is slightly higher than the apical efflux, which agrees well with almost the equal excretion of enalaprilat to urine and feces via bile.[128] Moreover, the hepatic influx of enalaprilat is 20-fold lower than its efflux clearances. The examples of losartan and enalapril clearly demonstrate that their hydrolysis results in metabolites with markedly

reduced passive permeability. However, efflux transporters increase the apparent permeability of these metabolites to a similar level as the passive permeability of the parent drug. Therefore, the hepatic transporters affect the extent of excretion of carboxylic acid metabolites over the basolateral membrane in the systemic circulation or over the apical membrane in bile.

Due to the membrane-barrier that restricts disposition of hydrophilic drug metabolites, the understanding of the uptake and efflux transporters in drug metabolite transport is an important and emerging topic.[129] Permeability-limited disposition does not only determine the systemic exposure and excretion routes for metabolites but also affects their intracellular concentrations, which might differ drastically from the systemic concentrations.[76] The intracellular hepatic concentrations are important factors when evaluating possible drug-drug interactions or hepatic toxicity of drug metabolites. For example, the evaluation of a CYP-mediated drug interaction based on the systemic concentration of a drug or drug metabolite might underestimate the magnitude of the interaction, if transporters contribute to the hepatic accumulation of the drug.[76,130] The concentration of a drug can be multiple folds higher in the hepatic tissue than in the systemic circulation. Although measuring hepatic concentrations in humans is infeasible, some data arise from positron-emission tomography imaging experiments. These data depict the magnitude of transporter-mediated hepatic accumulation of drugs in humans. For example, the human OATP substrates telmisartan and erlotinib concentrate over 10- and 40-fold, respectively, in the liver in comparison to blood in humans.[131,132] Data on the accumulation of drug metabolites in the liver are available only from animal studies. For example, sorafenib glucuronide reaches over 300-fold higher concentrations in the mouse liver than in plasma, which illustrates the effect of the membrane-barrier on disposition of hydrophilic drug metabolites in vivo.[133]

The effects of metabolism related changes and drug transporters on the excretion of drug metabolites are summarized in Table 4.

Table 4. *Disposition of drug metabolites derived from oxidation, conjugation and hydrolysis reactions. Typical metabolism related changes in the intrinsic molecular properties of a drug and effects of different factors on the disposition of these metabolites are indicated. The clearance terms are visualized in Figure 3 and defined in Equations 2-4.*[5,111-113,134]

Metabolic reaction	Typical biotransformation	Changes in intrinsic molecular properties	Disposition mechanism	Systemic exposure	Factors contributing to systemic clearance	Excretion route
Oxidation	Hydroxylation, dealkylation	Decrease of 0-2 logP units Increase of 10-20 Å^2 in PSA	Passive, if the parent is highly permeable (logD > 0, PSA < 75 Å^2)	Presumable and proportional to fraction metabolized via this route	2nd generation metabolism reactions, CL_r, f_u	Depends on 2nd generation metabolites, urine if high f_u
Conjugation, hydrolysis	Glucuronidation, sulfation, ester hydrolysis	Decrease of 2-5 logP units Increase of ~100 Å^2 PSA (glucuronic acid) A negative charge	Transporter mediated	Variable from negligible to high	Hepatic uptake and efflux transporters ($CL_{-influx}$, CL_{efflux} and $CL_{-efflux,bile}$) Renal transporters (CL_{-sec}) f_u	Urine if high hepatic $CL_{-efflux}$, low Cl_{influx} and high f_u Bile if high hepatic $CL_{-influx}$ and $CL_{-efflux,bile}$ Urine and bile if $CL_{-influx} < CL_{-efflux} \approx CL_{-efflux,bile}$

2.3 Transporter-mediated disposition of glucuronide metabolites

Glucuronide metabolites of drugs can be classified in either ECCS Class 3A or 3B based on their properties: a negative charge, low permeability and low metabolism. Thus, the MW (> 400 Da or < 400 Da) might affect their disposition via either urine or bile. However, glucuronides are formed intracellularly, which implicates that hepatic efflux transporters (CL_{efflux} and $CL_{efflux,bile}$) control the efflux from the hepatocytes, and only if CL_{efflux} is high, the hepatic CL_{influx} also contributes to the systemic exposure and clearance (Equation 4). In such cases, hepatic OATs and OATPs, in addition to the renal OATs, might play an important role in the systemic clearance of glucuronides (Table 2).

2.3.1 Efflux transporters in the excretion of glucuronides

In vivo evidence for the identification of individual transporters that affect the disposition of glucuronide metabolites in humans is scarce. The lack of in vivo reports is mostly due to difficulties in attributing any drug-drug interactions that involve glucuronide metabolites to the level of an individual efflux transporter. In addition, the effects of genotypes causing changes in the activity of efflux transporters in vivo are either rarely reported or have not correlated significantly with the disposition of glucuronides.[135] Finally, specific substrates among glucuronide metabolites or specific transporter inhibitors and inducers are currently unavailable.[69,125,136] Therefore, the in vivo investigations are highly limited to reveal any contribution of specific transporters to the disposition of glucuronides. An exception is the rare Dubin–Johnson syndrome that is caused by inactivating mutations in MRP2, which result in a 10- to 100-fold increase in the plasma concentration of bilirubin glucuronide that is normally excreted in bile by MRP2.[137] In addition, a non-coding variant of *ABCC2*, encoding MRP2, has been identified to correlate with the plasma levels of food-derived genistein and dihydrogenistein glucuronides in a study of 18 Japaneses.[138] However, the most of the direct evidence of human efflux transporters that contribute to disposition of glucuronide drug metabolites currently comes from in vitro experiments.

Strong evidence from in vitro human and in vivo animal studies supports that hepatic and intestinal MRP2 and MRP3 are the main transporters for glucuronide metabolites of drugs.[125,139,140] Growing evidence has revealed that MRP4 and BCRP are also involved in the transport of glucuronide conjugates.[120,123,125,133,141-145] P-gp, on the other hand, is unlikely to play a role in the disposition of conjugated drug metabolites such as glucuronides, even if this transporter is one of the most important drug transporter.[7,125] This negligible role of P-gp is mostly attributed to its repulsion of negatively charged and hydrophilic compounds, and specificity toward more lipophilic and cationic or neutral molecules.[7,146]

MRP2, MRP3, MRP4 and BCRP are important efflux transporters in drug disposition, as discussed in Section 2.1.4. The ABC transporter family contains almost 50 different proteins, although so far only proteins from the *ABCC* family and *ABCG2*, BCRP, have been identified to transport organic anions.[67] Thus, it is possible that other glucuronide efflux transporters remain to be found. In fact, knockout animal studies have revealed that Mrp2, Mrp3, Mrp4 and Bcrp do not always fully explain the excretion of glucuronides over the hepatic apical or basolateral membranes, which indicates that unknown transporters might be involved in the excretion of glucuronide metabolites.[133,147-149]

Most MRPs other than MRP2-4 are expressed ubiquitously in several tissues or at low mRNA levels in the liver.[85,150] However, some studies have reported rather high protein expression of MRP1, MRP5 and MRP6 in the liver.[83] No ideal substrate has been reported for MRP6 so far, and an unsuccessful attempt to study the transport of glucuronide conjugates by MRP6 is reported in a sub-study of this thesis (III). Therefore, the contribution of MRP6 to the transport of glucuronides is currently unknown.[151,152] MRP5 transports small anionic compounds, and methotrexate glucuronide has been reported as a low affinity substrate for this transporter.[153] However, it remains to be investigated whether MRP5 plays a role in the disposition of glucuronides. Finally, MRP1 transports glucuronide conjugates in vitro, and a study employing Mrp1-knockout mice reported that after oral administration of kaempferol its glucuronide conjugates were almost 10-fold less abundant in the systemic circulation in comparison to wild type mice.[154,155] The significance of MRP1, MRP5 and MRP6 in the disposition and transport of glucuronide metabolites of drugs should be clarified in future studies.

It is not a coincidence that MRPs transport glucuronide conjugates, since mostly only organic anions have been discovered so far as the substrates for these transporters.[85] In addition, MRP2 and MRP3 are expressed rather specifically and highly in the liver, in comparison to other tissues, and are localized to the opposite membranes of hepatocytes (Figure 5), which allows them to function as important physiological backup routes for each other.[83,139,140,150] The substrate specificity of MRP2, MRP3 and MRP4 overlaps considerably.[85,125,139] In particular, substrates of MRP2 and MRP3 overlap significantly, whereas MRP4 specifically transports cyclic nucleotides such as cAMP and cGMP that are rather small anionic compounds.[140,153] However, MRP4 is capable of transporting larger anions, such as estradiol-17β-glucuronide and dehydroepiandrosterone sulfate, similarly to MRP2 and MRP3. Therefore, it is impossible to draw conclusions on the factors that affect substrate recognition of these transporters. MRP2, but not MRP3, transports glutathione conjugates of drugs, indicating that MRP2 might transport larger MW compounds than MRP3 or MRP4.[85,125,139] Finally, BCRP accepts both sulfate and glucuronide conjugates as well as other versatile structures as substrates, but aromatic rings and their effect on flatness of the structure are important properties for them.[125,156] Thus, some substrates of MRPs might not be transported by BCRP. In addition, the charge of a compound is not crucial for BCRP in contrast to MRPs that tend to transport anionic compounds.[85,156]

2.3.2 Mechanistic studies on the effect of efflux transport on glucuronide disposition

Most of the evidence for glucuronide transporters and their mechanistic effects on glucuronide disposition has emerged from animal studies, particularly from knockout mice studies (summarized in Table 5). Additionally, multiple in vitro studies on human efflux transporters and their transport of glucuronide metabolites have been reported.[120,123,125,133,141-145] However, comprehensive studies that compare the transport properties of several efflux transporters side by side in the transport of several glucuronide conjugates are lacking. In particular, studies that accurately characterize transport kinetics of different human efflux transporters, which is crucial for estimating the fractional contribution of individual transporters, are rare in the current literature.[125,157]

Table 5. *Summary of animal studies that investigated disposition of glucuronide metabolites of drugs and drug-like compounds in Mrp2$^{-/-}$-, Mrp3$^{-/-}$-, Mrp4$^{-/-}$-and Bcrp$^{-/-}$-knockout mice or Mrp2-deficient rats. Values are related to the respective wild type strains and represent either the area under the curve (AUC) or single plasma concentrations (perfusate and plasma), cumulative excretion (bile) or hepatic absolute concentrations or relative amounts (liver), but not rates of excretion.*

Metabolite	Strain and administration route of the parent compound	Liver	Perfusate or Plasma	Bile
4-methylumbelliferone-G[148,149,158,159]	Mrp2-deficient rats (IPL)	NR	6× ↑	70×↓
	Bcrp$^{-/-}$ mice (ISPL)	2× ↑	↔	2×↓
	Mrp2$^{-/-}$ mice (ISPL)	↔	↔	2×↓
	Mrp3$^{-/-}$ mice (ISPL)	2× ↑	4×↓	5× ↑
	Mrp4$^{-/-}$ mice (ISPL)	↔	↔	↔
	Mrp3$^{-/-}$ mice (oral)	NR	4× ↓	NR
4-methyl-esculetin-G[160]	Bcrp1$^{-/-}$ mice (oral)	NR	2× ↑	NR
	Bcrp1$^{-/-}$ mice (i.v.)	NR	3× ↑	NR
	Mrp2$^{-/-}$ mice (oral)	NR	2× ↑	NR
	Mrp2$^{-/-}$ mice (i.v.)	NR	3× ↑	NR
Acacetin-G[161]	Bcrp$^{-/-}$ mice (oral)	↔	2× ↑	NR
	Mrp2$^{-/-}$ mice (oral)	5× ↑	6× ↑	NR
Acetaminophen-G[148,149,162,163]	Mrp2-deficient rats (IPL)	NR	2×↑	340×↓
	Mrp3$^{-/-}$ mice (i.v.)	20×↑	10×↓	10×↑
	Bcrp$^{-/-}$ mice (ISPL)	↔	↔	3×↓
	Mrp2$^{-/-}$ mice (ISPL)	3×↓	2×↑	3×↓
	Mrp3$^{-/-}$ mice (ISPL)	6×↑	5×↓	7×↓
	Mrp4$^{-/-}$ mice (ISPL)	↔	↔	↔
Bilirubin-G (endogenous)[164]	Mrp2$^{-/-}$ mice	NR	4× ↑	4× ↓
	Mrp3$^{-/-}$ mice	NR	↔	2× ↓
	Mrp2;Mrp3$^{-/-}$ mice	NR	↔	11× ↓
Bisphenol-A-G[158]	Mrp3$^{-/-}$ mice (oral)	NR	10× ↓	NR
Clopidogrel-G[165]	Mrp3$^{-/-}$ mice (oral)	liver/plasma ratio 11×↑	NR	NR
Diclofenac-G[147,166]	Bcrp1$^{-/-}$ mice (i.v.)	2× ↑	↔	2×↓
	Mrp2$^{-/-}$ mice (i.v.)	↔	6× ↑	2×↓
	Bcrp1;Mrp2$^{-/-}$ mice (i.v.)	2× ↑	24× ↑	4×↓
	Mrp3$^{-/-}$ mice (i.v.)	↔	2×↓	NR
	Bcrp1;Mrp3$^{-/-}$ mice (i.v.)	↔	2×↓	NR
	Mrp2;Mrp3$^{-/-}$ mice (i.v.)	↔	2×↓	NR
	Bcrp1;Mrp2; Mrp3$^{-/-}$ mice (i.v.)	3× ↑	↔	NR
	Bcrp1$^{-/-}$ mice (i.v.)	NR	3× ↑	↔
	Mrp3$^{-/-}$ mice (i.v.)	↔	6×↓	↔
E3040-G[158,167]	Mrp2-deficient rats (i.v.)	NR	12× ↑	4× ↓
	Mrp3$^{-/-}$ mice (oral)	NR	22× ↓	NR
Ethinylestradiol-G[168]	Bcrp1$^{-/-}$ mice (i.v.)	NR	2× ↑	NR
	Bcrp1$^{-/-}$ mice (oral)	NR	2× ↑	NR
	Mrp2$^{-/-}$ mice (i.v.)	NR	24× ↑	NR
	Mrp2$^{-/-}$ mice (oral)	NR	46× ↑	NR
Etoposide-G[169]	Mrp2$^{-/-}$ mice (i.v.)	2×↓	2× ↑	NR
	Mrp3$^{-/-}$ mice (i.v.)	↔	↔	NR
	Mrp2;Mrp3$^{-/-}$ mice (i.v.)	8× ↑	↔	NR
Ezetimibe-G[170]	Bcrp1$^{-/-}$ mice (oral)	↔	NR	↔
	Bcrp1;Mrp2$^{-/-}$ mice (oral)	70× ↑	270× ↑	40×↓
	Mrp2$^{-/-}$ mice (oral)	29× ↑	NR	2×↓
	Mrp3$^{-/-}$ mice (oral)	2×↓	14×↓ (portal vein)	3×↓
Gemfibrozil-G[158]	Mrp3$^{-/-}$ mice (oral)	NR	2× ↓	NR
Genistein-G[171,172]	Bcrp1$^{-/-}$ mice (oral)	NR	14× ↑	NR
	Bcrp1$^{-/-}$ mice (i.p.)	NR	3× ↑	NR

	Bcrp$^{-/-}$ mice (oral)	NR	7× ↑	NR
Harmol-G[148,149]	Bcrp$^{-/-}$ mice (ISPL)	NR	NR	16×↓
	Mrp2$^{-/-}$ mice (ISPL)	NR	↔	↔
	Mrp3$^{-/-}$ mice (ISPL)	NR	NR	↔
	Mrp4$^{-/-}$ mice (ISPL)	↔	↔	↔
Kaempferol-G[155]	Bcrp1$^{-/-}$ mice (oral)	NR	3× ↑	NR
	Mrp2$^{-/-}$ mice (oral)	NR	4× ↑	NR
Morphine-3-G[173,174]	Mrp2$^{-/-}$ mice (i.p.)	3×↓	2× ↑	100×↓
	Mrp3$^{-/-}$ mice (i.p.)	NR	14×↓	↔
	Mrp3; Mrp4$^{-/-}$ mice (i.p.)	NR	14×↓	NR
	Mrp2;Mrp3$^{-/-}$ mice (i.p.)	13× ↑	14×↓	100×↓
	Mrp3$^{-/-}$ mice (i.p.)	5× ↑	30×↓	5× ↑
PhIP-G[175]	Bcrp1;Mdr1a/b;Mrp2$^{-/-}$ mice (i.v.)	NR	2×↓	630×↓
Resveratrol-G[176,177]	Bcrp1$^{-/-}$ mice (oral)	↔	2× ↑	NR
	Mrp3$^{-/-}$ mice (oral)	2×↓	10×↓	NR
S-8921-G[178]	Mrp2-deficient rats (i.v.)	5× ↑	3× ↑	↔
	Bcrp$^{-/-}$ mice (i.v.)	NR	↔	↔
Sorafenib-G[133]	Mrp2$^{-/-}$ mice (oral)	3× ↑	350× ↑	12×↓
	Mrp3$^{-/-}$ mice (oral)	↔	↔	NR
	Mrp4$^{-/-}$ mice (oral)	↔	↔	NR
	Mrp3;Mrp4$^{-/-}$ mice (oral)	↔	↔	NR

E3040 = 6-hydroxy-5,7-dimethyl-2-methylamino-4-(3-pyridymethyl)benzothiazole, G = glucuronide, i.p. = intraperitoneal, IPL= isolated perfused liver, ISPL = in situ perfused liver, i.v. = intravenous, NR = not reported, PhIP = 2-Amino-1-methyl-6-phenylimidazo[4,5-b]pyridine, S-8921 = methyl 1-(3,4-dimethoxyphenyl)-3-(3-ethylvaleryl)-4-hydroxy-6,7,8-trimethoxy-2-naphthoate

The data in Table 5 clearly demonstrate that Mrp2, Mrp3 and Bcrp contribute to the disposition of glucuronide metabolites according to their location in hepatocytes and enterocytes, namely Mrp3 in the basolateral and Mrp2 and Bcrp in the apical membranes. Data in Table 5 indicate that Mrp4 does not contribute to the disposition of glucuronides of 4-methylumbelliferone, acetaminophen, harmol, morphine or sorafenib. Morphine-3-G is not an MRP4 substrate, whereas no data are available for the glucuronides of acetaminophen and harmol.[149,174] On the other hand, it was found within this thesis that 4-methylumbelliferone-G is even a better substrate for MRP4 than for MRP3 (I), although only the knockout of Mrp3 affected the hepatic excretion of 4-methylumbelliferone-G in mice (Table 5). Finally, MRP4 transports sorafenib-G similarly to MRP3 but the knockout of either transporter did not affect the excretion of this metabolite, indicating contribution of unknown basolateral transporters.[133]

The expression of MRP4 is low in the human liver and possibly also in the mouse liver.[77,83] Nevertheless, the decreased basolateral excretion of the sulfate metabolites of 4-methylumbelliferone, acetaminophen and harmol from the livers of Mrp4$^{-/-}$ mice in comparison to the wild type mice strongly suggests that a functional Mrp4 is present in the wild type mouse livers.[149] The discrepancies between the aforementioned in vivo mice and in vitro human studies remain to be explained, and the significance of hepatic MRP4 in the disposition of glucuronide metabolites should be studied in more detail. In addition, only a few studies have investigated interspecies differences between human and animal efflux transporters, and thus no conclusions can be made about the similarities in the substrate preference between mouse and human MRPs.[179]

Another clear conclusion from the data in Table 5 is the notable overlapping substrate specificity between Mrp2 and Mrp3. The absence of hepatic Mrp2 shifts the excretion pathway from biliary to systemic that is mostly mediated by Mrp3. For example, the knockout of Mrp2 doubles the systemic exposure of etoposide-G and decreases its hepatic concentration to half, while the Mrp3-knockout does not affect the respective values (Table 5).[169] The double knockout of Mrp2 and Mrp3, on the other hand, increases the hepatic amount of etoposide-G to an eight-

fold higher level than in the wild type or Mrp3 knockout mice, while this knockout does not affect the systemic concentration. Such observations clearly demonstrate the compensating roles of Mrp2 and Mrp3 for each other. If both transporters are absent, the intracellular amounts of glucuronide metabolites increase at least several fold, depending on other transporters that carry the metabolites. It should be noted that upregulation of hepatic Mrp3 is usually observed in Mrp2-knockout mice and Mrp2-deficient rats.[159,169] This upregulation may partly explain the higher basolateral excretion in knockout animals, or lower hepatic concentrations as in the case of etoposide-G, compared to wild type animals. Finally, results from animal knockout studies should not be translated directly to humans or interpreted quantitatively, because of the upregulation of some transporters or differences in their basal expression levels and substrate specificities in animals in comparison to human tissues.[157] Nevertheless, knockout studies provide mechanistic understanding on the effect of efflux transporters and to what extent they affect glucuronide metabolite disposition.

Efflux transporter knockout studies may help deduce the fraction excreted (f_e) parameter for a specific transporter. Furthermore, f_e can be used to estimate the effect of a putative transporter inhibitor (I with an inhibition constant K_i) on the relative change of metabolite exposure (foldΔ) in blood, tissue or bile according to Equation 5.[180] If the whole pathway is knocked out ([I]>>K_i), the subtracted form of Equation 5 can be employed. Knockout of pathways that have an f_e value ≤ 0.5 or their full inhibition would lead to a maximum of two-fold change in the exposure, not more. Because hepatic efflux transporters have considerable overlapping substrate specificities in the transport of glucuronide metabolites, Equation 5 might explain why drug-drug interactions or genotype effects have not been reported at the efflux transport level in humans. Furthermore, determination of f_e to different human efflux transporters would demand comprehensive studies with human transporters in vitro and extrapolation of in vitro expression levels of transporters to in vivo with the aid of proteomics data.[76] Thus, better understanding of different human efflux transporters in vitro is warranted.

(5) $$fold\Delta = \frac{1}{\frac{f_e}{(1 + [I]/K_i)} + 1(1 - f_e)}$$, if [I]>>K_i then $fold\Delta = \frac{1}{1-f_e}$

2.3.3 Interplay of uptake and efflux transport in the disposition of glucuronide metabolites

The systemic clearance of glucuronide metabolites is controlled by the mechanisms presented in Equation 4. The metabolism of glucuronide conjugates is usually negligible, although acyl glucuronides with an ester bond might be prone to esterase-mediated cleavage.[19,181] In addition, $CL_{passive}$ of glucuronides is low and therefore Equation 4 is simplified to Equation 6.

(6) $$CL_{int,h} = \frac{CL_{influx} \times CL_{efflux,bile}}{(CL_{efflux} + CL_{efflux,bile})}$$

Efflux transporters are rarely specific, as discussed in Section 2.3.1. Furthermore, both the apical and basolateral transporters exhibit substantially overlapping substrate specificity, implicating that in most cases both CL_{efflux} and $CL_{efflux,bile}$ contribute to the hepatic excretion. Thus, the systemic exposure of a glucuronide metabolite is dependent on the f_e values of both the apical and basolateral efflux transporters. However, as Equation 6 indicates, CL_{influx} is the major factor

contributing to $CL_{int,h}$, and thus the net secretion from the liver to the systemic circulation is related to CL_{influx}/CL_{efflux}. Together, the disposition of glucuronide metabolites is not only mediated by the efflux transporters but also by the uptake transporters of which the most important in the liver are OATP1B1, OATP1B3, OATP2B1, OAT2 and the emerging OAT7, as discussed in Section 2.1.4. Finally, the renal uptake transporters, particularly OAT1 and OAT3, contribute to CL_{sec}, and if CL_r is high or similar to the hepatic clearance, the renal transporters should also be considered in the disposition of glucuronides.

A representative example of the impact of different mechanisms on the disposition of a glucuronide conjugate is provided by the mechanistic studies with E3040, a small molecular inhibitor of 5-lipoxygenase and thromboxane A2 synthetase, and its glucuronide metabolite, E3040-G, in rats. E3040-G is a substrate for both Mrp2 and Mrp3 (Table 5), and hepatic uptake transporters have a major contribution to its uptake into rat hepatocytes.[182] After intravenous administration of E3040 to rats, 95% of the formed glucuronide was excreted in bile and only 5% in urine (Figure 6).[167] The same amount of the glucuronide metabolite was excreted in bile also after intravenous administration of E3040-G, and its CL_r was only 5% of the total plasma clearance, while no metabolism was found.[182] This indicates that the hepatic uptake is responsible for 95% of the total plasma clearance of E3040-G. Mrp2-deficient rats had 71-fold lower $CL_{efflux,bile}$ and similar CL_r in comparison to the wild type rats, while the cumulative amount of biliary and urinary excreted E3040-G was only 5-fold lower and 15-fold higher, respectively.

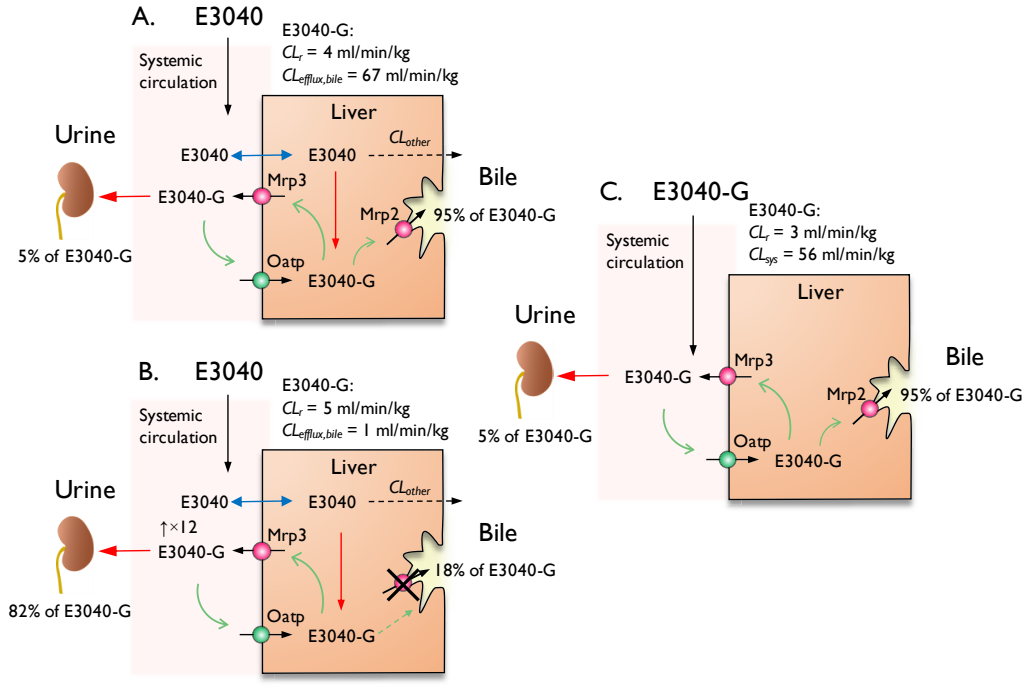


Figure 6 *Schematic presentation of the disposition of E3040-G in rats. E3040 or E3040-G were administered intravenously to wild type (A, C) or Mrp2-deficient rats (B). Cl_{other} represents other metabolic clearances, which account for about 70% of the total clearance in the cases of A and B.*[158,167,182]

The data of E3040-G elegantly illustrate that the systemic exposure and subsequent urinary excretion is not only determined by the relative f_e of the hepatic basolateral and apical efflux transporters but by the net clearance, CL_{influx}/CL_{efflux}, over the basolateral membrane. If the net clearance is high, even low $CL_{efflux,bile}$ will significantly contribute to the cumulative biliary excretion of the compound (Figure 6).

The interaction of glucuronide metabolites with hepatic OATP1B1, OATP1B3, OATP2B1 and OAT2, as well as renal OAT1 and OAT3 is an emerging research topic, although the currently available data are still scarce.[120,123,124,129,183,184] However, similarly to knockout of efflux transporters in mice, knockout of the homological mouse transporters of hepatic OATPs have been developed, and a few studies have investigated disposition of glucuronide metabolites in these mice. It should be noted that direct orthologues of human OATP1B1 and OATP1B3 are absent from the mouse liver.[179]

Oral administration of regorafenib to Oatp1b2$^{-/-}$ mice resulted in a nine-fold higher plasma AUC of regorafenib glucuronide in comparison to wild type mice.[185] Furthermore, the plasma AUC of sorafenib glucuronide was increased 72-fold in Oatp1a/1b$^{-/-}$ mice in comparison to wild type mice after administration of oral sorafenib.[133] Remarkably, further knockout of Mrp2 in Oatp1a/1b$^{-/-}$ mice resulted in a total of 900-fold higher plasma AUC of sorafenib glucuronide in comparison to wild type mice. Moreover, the sorafenib-G concentration ratio of liver to plasma was reduced from 350 in wild type mice to about 1 in Mrp2;Oatp1b2$^{-/-}$ mice. These observations indicate that in the absence of CL_{influx}, sorafenib-G is completely transported via the hepatic basolateral membranes to the systemic circulation and no accumulation of the metabolite into the liver occurs. These studies further confirm the mechanistic importance of the hepatic CL_{influx} in determining the systemic exposure of glucuronide metabolites.

Hepatic uptake does not only affect the hepatic derived glucuronides but also glucuronides formed in enterocytes and excreted over the basolateral membranes to blood after oral drug administration. Zeng and coworkers infused glucuronides of several flavonoids, including apigenin, biochanin A, chrysin and genistein, into the portal vein of rats and found that the liver extracted 60-100% of the glucuronides, which indicates excessive transporter-mediated uptake into liver.[186] Finally, some glucuronide metabolites have been identified as OAT substrates. For example, cabotegravir and morinidazole glucuronides are OAT3 substrates and diclofenac glucuronide is a substrate for OAT1, OAT3 and OAT4.[123,183,184] These three transporters may contribute to CLr of the aforementioned glucuronide metabolites. In addition, hepatic OAT2 and OAT7 were only recently identified to contribute to the active uptake of anionic drugs into the liver.[74] Thus, future research of their impact on glucuronide metabolites is warranted.

3 Aims of the study

The main aims of this thesis were to identify molecular mechanisms that control the excretion of drug metabolites from the human liver and intestine, and whether these mechanisms can explain the extent of systemic exposure, urinary excretion and biliary excretion of hydrophilic drug metabolites. Specific aims were set to study the activity of human hepatic and intestinal efflux transporters MRP2, MRP3, MRP4, BCRP and P-gp in the transport of glucuronide conjugates that are typical hydrophilic drug metabolites, which require carrier-mediated mechanisms for their excretion from tissues.

For each sub-study, a set of glucuronide metabolites of drugs or drug-like compounds was selected and their transport by human MRP2, MRP3, MRP4, BCRP and P-gp was compared in the activity and transport kinetic assays:

I Planar ring containing and mostly small MW glucuronides, including 1-hydroxypyrene, 1-naphthol, 4-methylumbelliferone glucuronides and two glucuronides of enantiomeric R- and S-propranolol.

II Small MW glucuronides of nicotine and of its oxidative metabolites cotinine and *trans*-3'-hydroxycotinine.

III High MW glucuronides of estrogens including estradiol, estriol and estrone.

IV High MW glucuronides of androgens including dehydroepiandrosterone, epitestosterone, testosterone as well as the glucuronides of its metabolites androsterone and etiocholanolone.

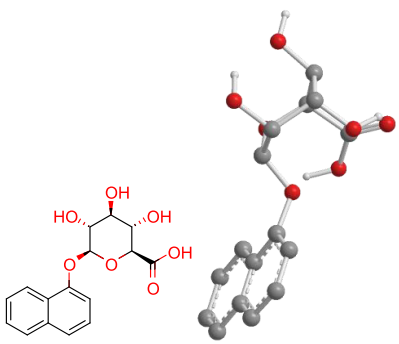
4 Materials and Methods

The key materials and methods of this thesis are presented in this section. Full details are described in Publications I-IV.

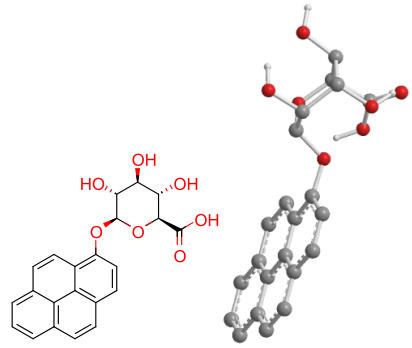
4.1 Materials

The glucuronides investigated in this thesis, including their key intrinsic molecular properties MW, $logD_{7.4}$ and PSA, which were calculated in MarvinSketch (version 19.15, ChemAxon Ltd.), are presented in Table 6. The 3D conformations were generated in Chem3D and the MM2 function was used to minimize the energy of structures (version 17.0.0.206, PerkinElmer Informatics Inc.).

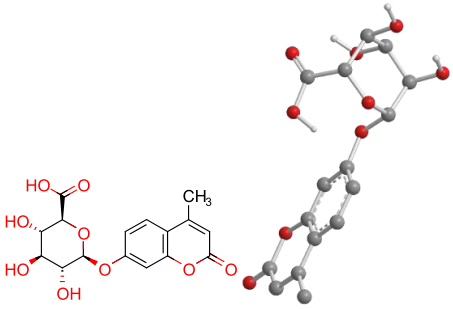
Table 6. *Molecular structures and the intrinsic molecular properties of the glucuronides investigated in this thesis. Hydrogen atoms, except polar hydrogens, are omitted for clarity. G stands for glucuronide.*



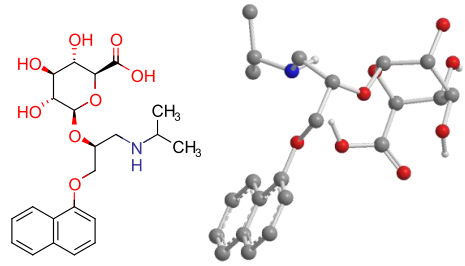
1-naphthol-G
MW = 320.3 Da, $logD_{7.4}$ = -2.64, PSA = 116 Å²



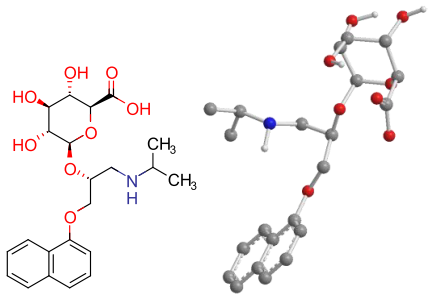
1-hydroxypyrene-G
MW = 394.4 Da, $logD_{7.4}$ = -1.24, PSA = 116 Å²



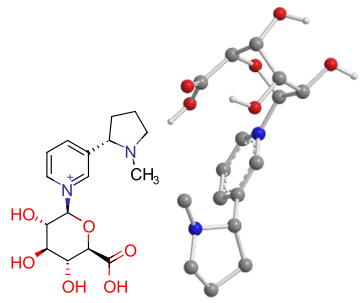
4-methylumbelliferone-G
MW = 352.3 Da, $logD_{7.4}$ = -3.65, PSA = 143 Å²



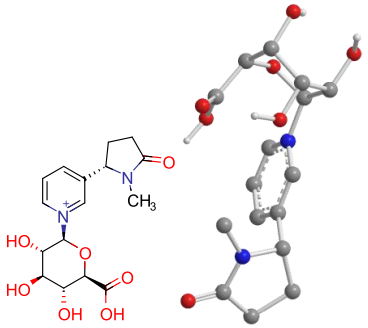
R-propranolol-G
MW = 435.5 Da, $logD_{7.4}$ = -1.34, PSA = 138 Å²



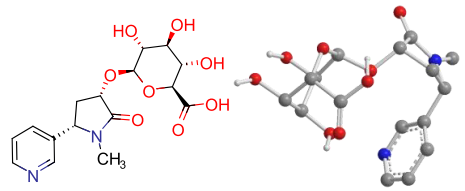
S-propranolol-G
MW = 435.5 Da, $\log D_{7.4}$ = -1.34, PSA = 138 Å²



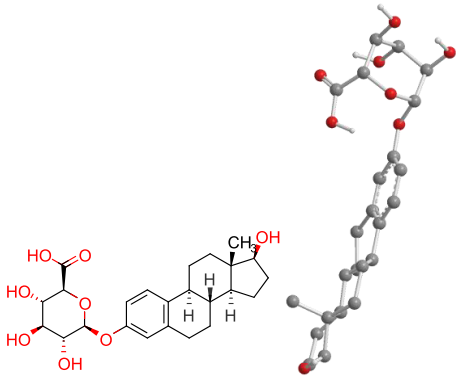
Nicotine-G
MW = 339.4 Da, $\log D_{7.4}$ = -5.29, PSA = 114 Å²



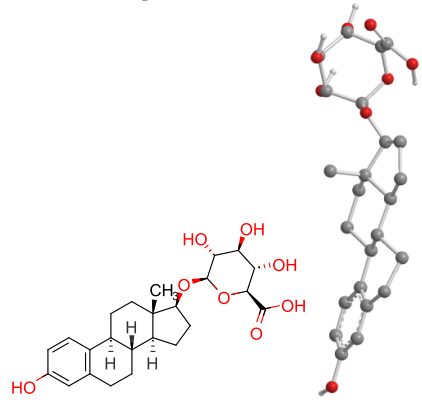
Cotinine-G
MW = 353.4 Da, $\log D_{7.4}$ = -6.06, PSA = 131 Å²



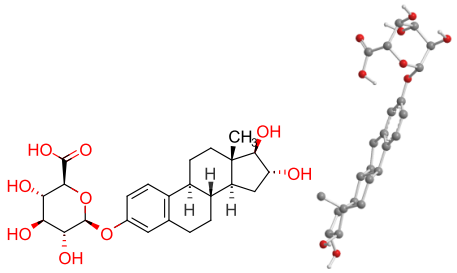
***Trans*-3'-Hydroxycotinine-G**
MW = 368.4 Da, $\log D_{7.4}$ = -5.47, PSA = 150 Å²



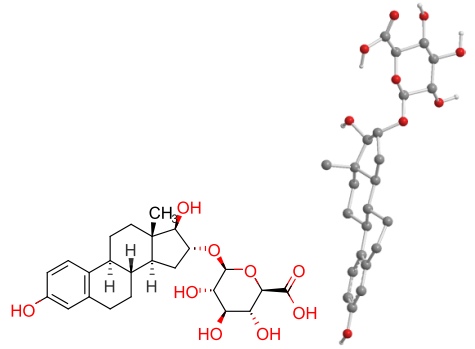
Estradiol-3-G
MW = 448.5 Da, $\log D_{7.4}$ = -1.63, PSA = 137 Å²



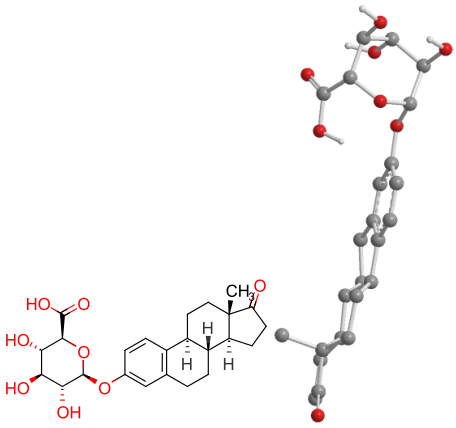
Estradiol-17β-G
MW = 448.5 Da, $\log D_{7.4}$ = -1.04, PSA = 137 Å²



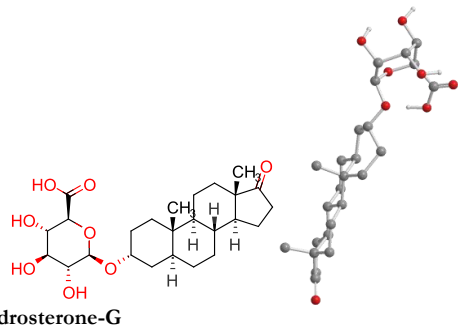
Estriol-3-G
MW = 464.5 Da, $\log D_{7.4}$ = -2.70, PSA = 157 Å²



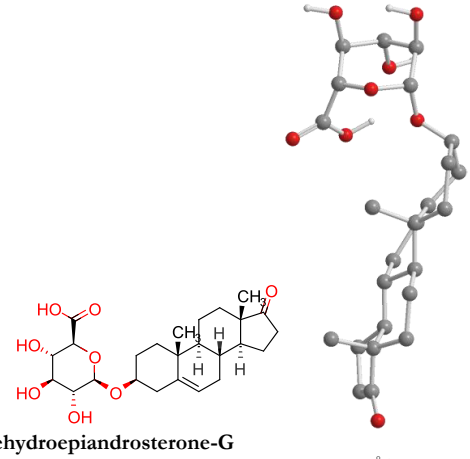
Estriol-16α-G
MW = 464.5 Da, $\log D_{7.4}$ = -2.17, PSA = 157 Å²



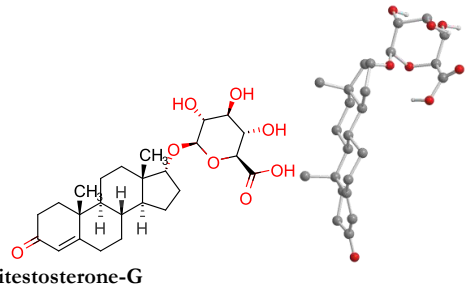
Estrone-3-G
MW = 446.5 Da, $logD_{7.4}$ = -1.06, PSA = 134 Å²



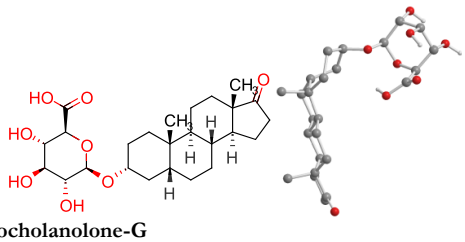
Androsterone-G
MW = 446.6 Da, $logD_{7.4}$ = -1.07, PSA = 134 Å²



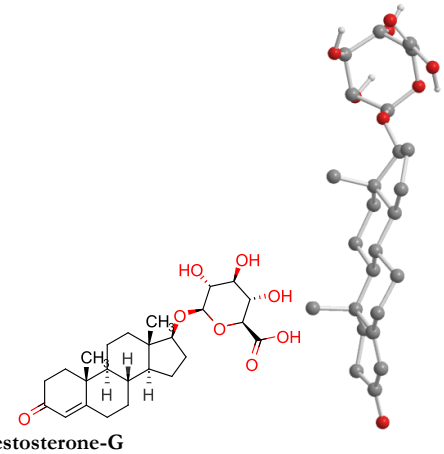
Dehydroepiandrosterone-G
MW = 465.6 Da, $logD_{7.4}$ = -1.48, PSA = 134 Å²



Epitestosterone-G
MW = 464.6 Da, $logD_{7.4}$ = -1.42, PSA = 134 Å²



Etiocholanolone-G
MW = 466.6 Da, $logD_{7.4}$ = -1.07, PSA = 134 Å²



Testosterone-G
MW = 464.6 Da, $logD_{7.4}$ = -1.42, PSA = 134 Å²

Androsterone-G, estradiol-17β-G, estriol-3-G, estriol-16α-G, dehydroepiandrosterone-G, testosterone-G, 1-naphthol-G and 4-methylumbelliferone-G were from Sigma-Aldrich, whereas etiocholanolone-G was from Steraloids. Estradiol-3-G was from Cayman Chemical, whereas estrone-3-G, nicotine-G, cotinine-G and *trans*-3'-hydroxycotinine-G were from Toronto Research Chemicals. N-methyl-quinidine was acquired from Solvo Biotechnology. The glucuronide of 1-hydroxypyrene was previously synthesized in-house.[187] Epitestosterone-G was biosynthesized within this study using the human UGT2B7 as the catalyst, whereas R- and S-propranolol-G were biosynthesized within this study using the human UGT1A10 and UGT1A9, respectively, as the catalysts. The details of biosyntheses are reported in Publications I and IV.

Water-soluble cholesterol- RAMEB (randomly substituted methyl-β-cyclodextrin) complex was from Cyclolab Ltd. The source of protease inhibitors was typically cOmplete Ultra EDTA-free protease inhibitor cocktail tablets from Roche.

4.2 Preparation of membrane vesicles for the efflux transport assays

Human efflux transporter cDNA-containing baculoviruses were employed to infect Sf9 insect cells for the production of recombinant transporter proteins. The construction and preparation of recombinant baculoviruses for human MRP2, BCRP and P-gp has been reported earlier.[188-190] The preparation methods of baculoviruses for MRP3 and MRP4 are reported in Publication I. For one membrane vesicle batch of a transporter, a suspension culture of Sf9 cells, 800 to 1600 ml at a density of 1.6×10^6 cells per milliliter, were infected with 0.5 to 2.5 µl of the third passage (P3) baculovirus, containing the transporter cDNA, per milliliter of culture. The amount of virus employed for the cell infection was optimized to achieve high expression of each transporter. After the infection, the cells were cultured for 3 days, harvested and the cell pellets were stored at -20 °C.

For the control preparations (CTRL), cells were infected with either empty baculovirus (I and III) or baculovirus containing cDNA that encodes an inactive mutant of MRP3 (II and IV). The construction of baculovirus for the inactive MRP3 mutant, the expression of the protein and verifying its inactivity are reported in Publication II.

The protocol for the preparation of inside-out membrane vesicles was adapted from Chu and coworkers.[191] For the membrane vesicle preparation, a cell pellet from 100 ml culture was placed on ice and resuspended in 15 ml of cold buffer (50 mM Tris hydrochloride pH 7.0 and 300 mM mannitol) containing protease inhibitors. After 45 min incubation on ice, the cells were collected by 10 min centrifugation at 1200 g and resuspended in 10 ml of cold TME-buffer (50 mM Tris hydrochloride pH 7.0, 50 mM mannitol, and 2 mM EGTA), homogenized by 40-80 strokes with a Dounce homogenizer with pestle B and incubated on ice for 60 min. Cell debris was removed by centrifugation for 10 min at 1200 g and the resulting supernatant was subjected to 75 min centrifugation at 100 000 g. The resulting pellet contained the membrane fraction of cells, which was resuspended in about 1 ml of TME-buffer and passed 20 times through a 27-gauge needle to form membrane vesicles. Finally, the total protein concentration of membrane vesicles was adjusted to 5 mg/ml with TME-buffer, followed by a snap-freeze in liquid nitrogen and transfer to -80 °C for storage.

BCRP, P-gp and their control ($CTRL^{+C}$) vesicles were supplemented with additional cholesterol to enhance their transport activity as reported.[192,193] For cholesterol supplementation, the membrane fraction was resuspended in 1-2 ml of TME-buffer with 2.5 mM

cholesterol/RAMEB complex, and this suspension was incubated for 20 min on ice with gentle mixing. Next, the suspension was diluted with TME-buffer and centrifuged again for 75 min at 100 000 g, after which it was processed similarly to the other preparations.

4.3 Vesicular transport assays and data analyses

The vesicle-based transporter assay was employed to study efflux transport in this thesis.[136] For the assay, transporter vesicles were thawed at +37 °C, after which 20-50 µg of total vesicle protein per sample was mixed with transport assay buffer (60 mM KCl, 40 mM MOPS adjusted to pH 7.0 with Tris-HCl and 6 mM $MgCl_2$) including a pre-determined concentration of the compound to be studied, in a total volume of 50 µl. The stocks of glucuronide conjugates (Table 6) were prepared in either DMSO or water (nicotine glucuronides) at 50 mM, and the maximum final concentration of DMSO in the assays was 1%. Samples were prepared on a 96-well plate on ice and then placed on a 37 °C shaker at 500 rpm for 10 min.

The transport reactions were initiated by the addition of 25 µl of transport assay buffer containing 6 mM Mg-ATP (+ATP samples) or plain buffer (-ATP samples). Final concentrations of the assay components in the transport reaction were 60 mM KCl, 40 mM MOPS, 6 mM $MgCl_2$, 4 mM Mg-ATP (only +ATP samples) and the final volume was 75 µl. The final concentrations of the glucuronides ranged from 1 to 10 µM in the single concentration assays, as indicated in the figure legends, or were several different concentrations in the case of the transport kinetic assays. The transport reactions were incubated at 37 °C and 500 rpm shaking for a pre-determined time (between 1 and 10 min, indicated in the figure legends), before reactions were terminated by the addition of 200 µl cold stop buffer (40 mM MOPS adjusted to pH 7.0 and 70 mM KCl). Finally, samples were transferred to a glass fiber filter plate (pore size 1.0 µm, from Merck Millipore) that was kept under vacuum, and an additional 200 µl of cold stop buffer was applied five times into wells of the filter plate.

After the samples had been washed, the filter plate was kept at room temperature for 1-2 hours for drying. To disrupt the vesicles and elute the compounds, 100 µl of elution solvent was applied into each well and the plate was placed on a shaker adjusted to 250 rpm for 30-60 min. Finally, sample eluates were collected to a new well plate by centrifuging the filter plate on the top of a collection plate for 2 min at 3 000 g. The elution solvents were 50% methanol containing 0.1% formic acid (I), 50% acetonitrile containing 0.1% formic acid (III), 75% methanol containing 0.1% formic acid (IV), or 70% acetonitrile containing 10 mM ammonium acetate (II). In case of analysis with ultra-performance liquid chromatography connected to triple quadrupole mass spectrometry (UPLC-MS/MS), the elution solvent also contained an internal standard that was a glucuronide compound of a similar structure to the analyte (II-IV).

Each assay included triplicate samples for every data point and the mean value from a single experiment is presented with ± SD in the Results section. The transport kinetics included seven to eight different concentrations around the estimated K_m value, and the transport reaction times were selected based on the pre-determined linear ranges of the transport (data reported in I-IV). To exclude solubility problems or other artifactual factors during the assay, the -ATP data were examined by plotting them against the concentrations of the compound investigated and a linear fit was ensured, which mostly resulted in R^2 values over 0.99, but in a few cases R^2 was 0.97-0.98. The maximum determined solubility for androgen and estrogen glucuronides was 500 µM.

The ATP-dependent transport kinetic data presented in the Results section were derived by subtracting the results of -ATP samples from +ATP samples, and the data represent mean ± combined SD of the subtracted values. The resulting transport rate (v) was fitted to either the Michaelis-Menten equation (Equation 7) or Hill's equation (Equation 8) with least squares fit. V_{max}, [S], K_m, h and S_{50} are the maximum transport rate, substrate concentration, the Michaelis constant, the Hill coefficient and the concentration producing half-maximal transport rate, respectively.

(7) $$v = \frac{V_{max} \times [S]}{K_m + [S]}$$

(8) $$v = \frac{V_{max} \times [S]^h}{S_{50}{}^h + [S]^h}$$

4.4 Analytical methods

High performance liquid chromatography (HPLC) connected to a fluorescence (FL) detector was employed to quantify 1-naphthol-G, 1-hydroxypyrene-G, 4-methylumbelliferone-G, R- and S-propranolol-G, as well as estradiol-17β-G. UPLC-MS/MS was employed for the quantification of the other compounds. External standard quantification was used for HPLC-FL analyses, whereas the internal standard method was employed for UPLC-MS/MS analyses. The HPLC-FL methods are reported in Publication I and UPLC-MS/MS methods in Publications II-IV. The HPLC-FL method for the analysis of N-methyl-quinidine has been reported elsewhere.[190]

5 Results

This section summarizes the main results of Publications I-IV.

5.1 Functionality of the efflux transporters in this thesis

The correct transport activity of the human efflux transporters in the membrane vesicles for in vitro assays in this thesis was verified by employing the known MRP2-4 and BCRP substrate estradiol-17β-G and the P-gp substrate N-methyl-quinidine (Figure 7).

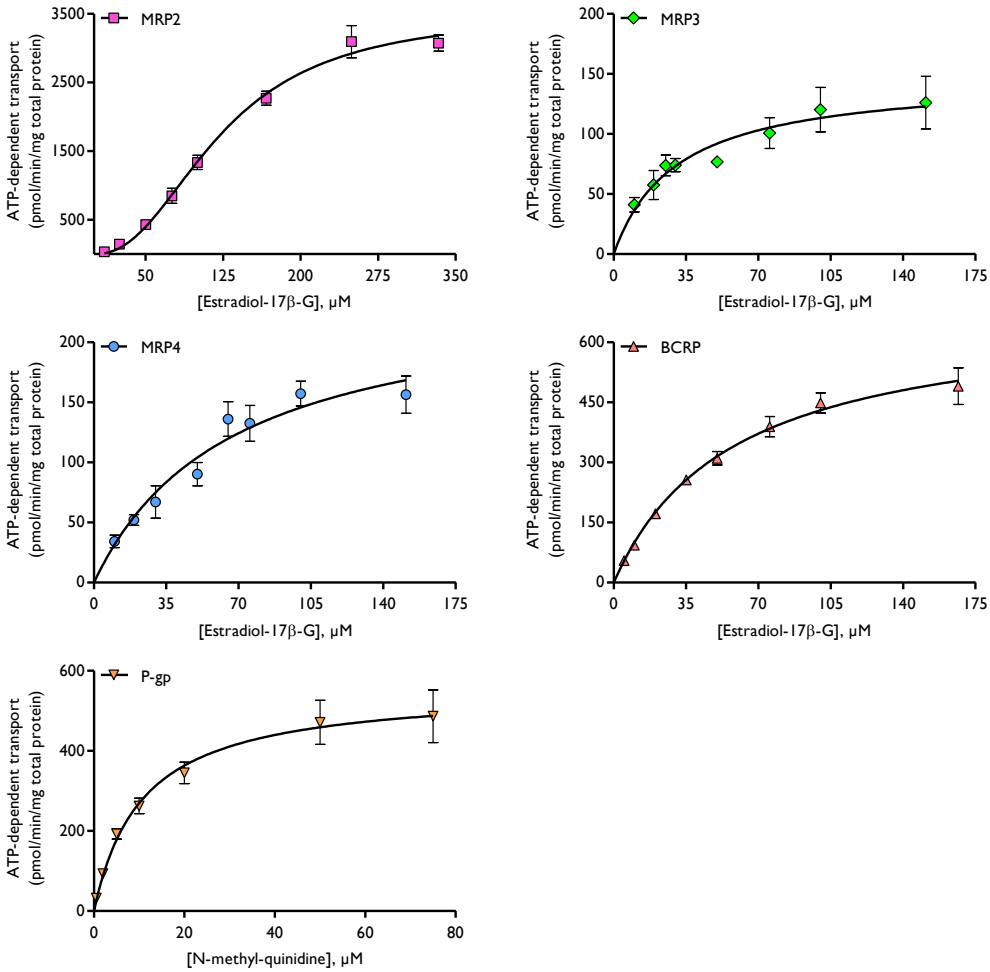


Figure 7 *The transport kinetics of MRP2, MRP3, MRP4, BCRP and P-gp. Well-characterized substrates of these transporters were employed. The transport reaction time was either 3 min (MRP3, MRP4 and P-gp) or 6 min (MRP2 and BCRP). The solid lines represent fitting of the data. The P-gp transport data of N-methyl-quinidine have been reported earlier.*[194]

MRP2-mediated transport kinetics of estradiol-17β-G yielded a distinct sigmoidal curve, whereas the other transporters followed Michaelis-Menten kinetics (Figure 7). The type of transport kinetics as well as the derived kinetic values are in line with the reported literature values (Table 7), which verifies the correct functionality of the efflux transporters employed in the vesicular transport assays of this thesis.

Table 7. *The derived kinetic constants for the data in Figure 7. The data were fitted to the Michaelis-Menten equation, except in the case of MRP2 Hill's equation was employed.*

Transporter	K_m (95% CI), μM	V_{max} (95% CI), pmol/min/mg total protein	Literature values[192,193,195-197]
		Estradiol-17β-G	
MRP2	S_{50} = 124 (108-139) h = 2.3 (1.9-2.7)	3520 (3180-3850)	S_{50} = 105 μM, h = 2.9, V_{max} = ~3000 pmol/min/mg total protein
MRP3	30 (18-43)	148 (126-169)	K_m = 26 μM, V_{max} = 76 pmol/min/mg total protein
MRP4	67 (38-95)	243 (194-292)	K_m = 30 μM, V_{max} = 102 pmol/min/mg total protein
BCRP	58 (46-69)	678 (621-735)	K_m = 45 μM, V_{max} = 700 pmol/min/mg total protein
		N-methyl-quinidine	
P-gp	11 (7.7-14)	557 (509-604)	K_m = 4 μM, V_{max} = 657 pmol/min/mg total protein

5.2 Transport of small glucuronides (I)

The transport of glucuronides of 4-methylumbelliferone, 1-naphthol, 1-hydroxypyrene, R- and S-propranolol by MRP2-MRP4 and BCRP was assayed at a single substrate concentration (Figure 8). The results indicated that MRP4 is highly active in the transport of the three glucuronides with planar aromatic rings, namely 4-methylumbelliferone-G, 1-naphthol-G, 1-hydroxypyrene-G, whereas propranolol glucuronides were transported only by MRP3, although at low rates. Furthermore, the transport of 4-methylumbelliferone-G, 1-naphthol-G and 1-hydroxypyrene-G was observed by MRP3 and BCRP, even though the control vesicles for BCRP were active in the case of 4-methylumbelliferone-G and 1-naphthol-G. The MRP2-mediated transport of 4-methylumbelliferone-G, 1-naphthol-G and 1-hydroxyprene-G was too low to be characterized in the transport kinetic assays. In the case of BCRP with 4-methylumbelliferone-G or 1-naphthol-G, the difference in the transport activity between control and BCRP vesicles was too low to reveal accurately the real contribution of BCRP, particularly at a higher concentration or in the presence of a specific BCRP inhibitor (data shown in Publication I).

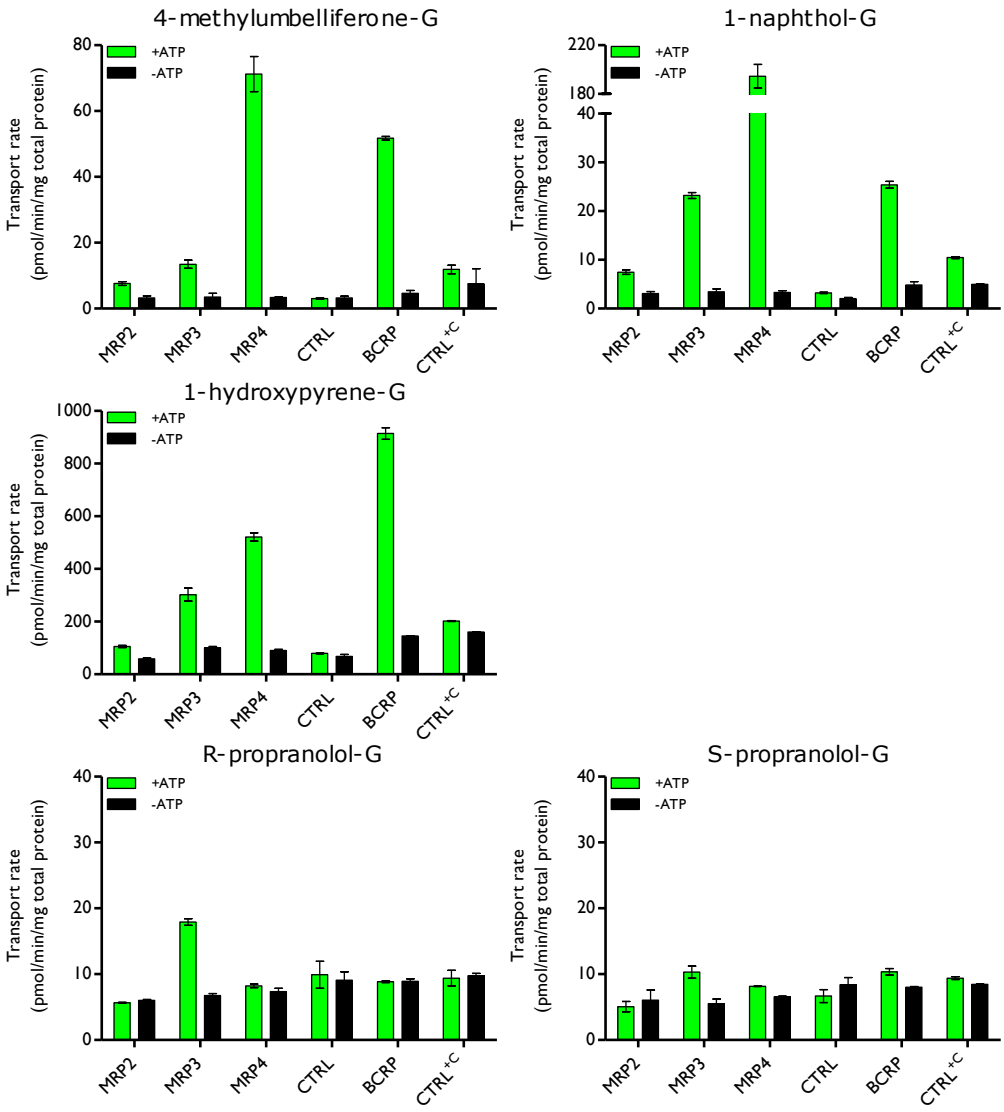


Figure 8 *Single concentration transport of the small glucuronides. The concentration of the glucuronides was 10 µM and the transport reaction incubation time was 5 min.*

Results from the transport kinetic experiments with MRP3, MRP4 and BCRP (Figure 9) were fitted to the Michaelis-Menten equation (Table 8). The MRP4-mediated transport of 4-methylumbelliferone-G, 1-naphthol-G and 1-hydroxypyrene-G occurred at higher V_{max} and lower K_m values in comparison to MRP3, whereas the MRP3-mediated transport kinetics of propranolol glucuronides resulted in high K_m values (Table 8). A clear difference was observed between R- and S-propranolol-G in the transport kinetic study (Figure 9) similarly to the single substrate concentration assay (Figure 8), namely that the R-isomer is a better substrate for MRP3 than the S-isomer. The BCRP-mediated transport of 1-hydroxypyrene-G occurred at a similar K_m value to MRP3 and MRP4 but at a much higher V_{max} value.

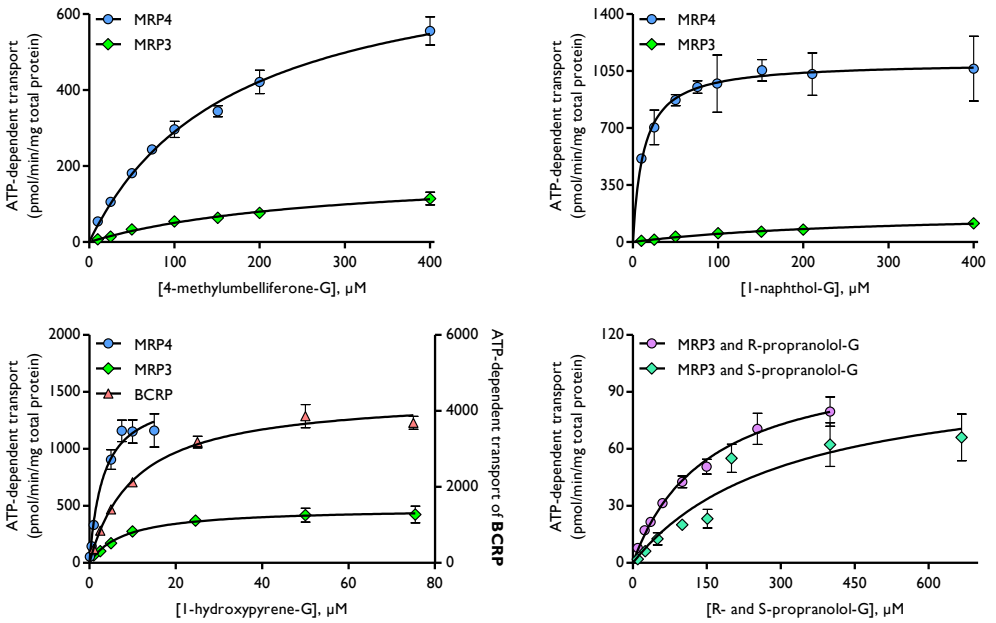


Figure 9 *The transport kinetics of the small glucuronides. The transport reaction times were 3 min for MRP4 and 5 min for MRP3 in the transport of 4-methylumbelliferone-G, 10 min for MRP3 in the transport of propranolol glucuronides and 1 min in the other cases. The solid lines represent fitting of the data to the Michaelis-Menten equation.*

Table 8. *The derived kinetic constants for the transport kinetic data in Figure 9.*

Substrate	K_m (95% CI), µM	V_{max} (95% CI), pmol/min/mg total protein
	MRP3	
1-naphthol-G	98.2 (45.0-151)	192 (150-234)
1-hydroxypyrene-G	8.08 (5.64-10.5)	481 (440-523)
4-methylumbelliferone-G	278 (178-377)	191 (153-229)
R-propranolol-G	154 (117-192)	110 (98.0-122)
S-propranolol-G	315 (108-522)	104 (108-522)
	MRP4	
1-naphthol-G	12.7 (7.64-17.7)	1110 (1030-1180)
1-hydroxypyrene-G	3.21 (1.90-4.53)	1510 (1320-1690)
4-methylumbelliferone-G	168 (139-197)	778 (712-844)
	BCRP	
1-hydroxypyrene-G	10.5 (8.55-12.3)	4430 (4180-4670)

5.3 Transport of the glucuronide metabolites of nicotine (II)

Nicotine-G and the glucuronides of two oxidation metabolites of nicotine, cotinine-G and *trans*-3'hydroxycotinine-G, were assayed in the vesicular transport assay (Figure 10). None of the transporters studied were active toward nicotine-G or cotinine-G, whereas only MRP3 actively transported *trans*-3'hydroxycotinine-G. The data from MRP3 transport kinetics of *trans*-3'hydroxycotinine-G were fitted to the Michaelis-Menten equation, which resulted in an estimated K_m value of 728 (411-1050 95% CI) µM and V_{max} of 501 (383-619 95% CI) pmol/min/mg total protein (Figure 10).

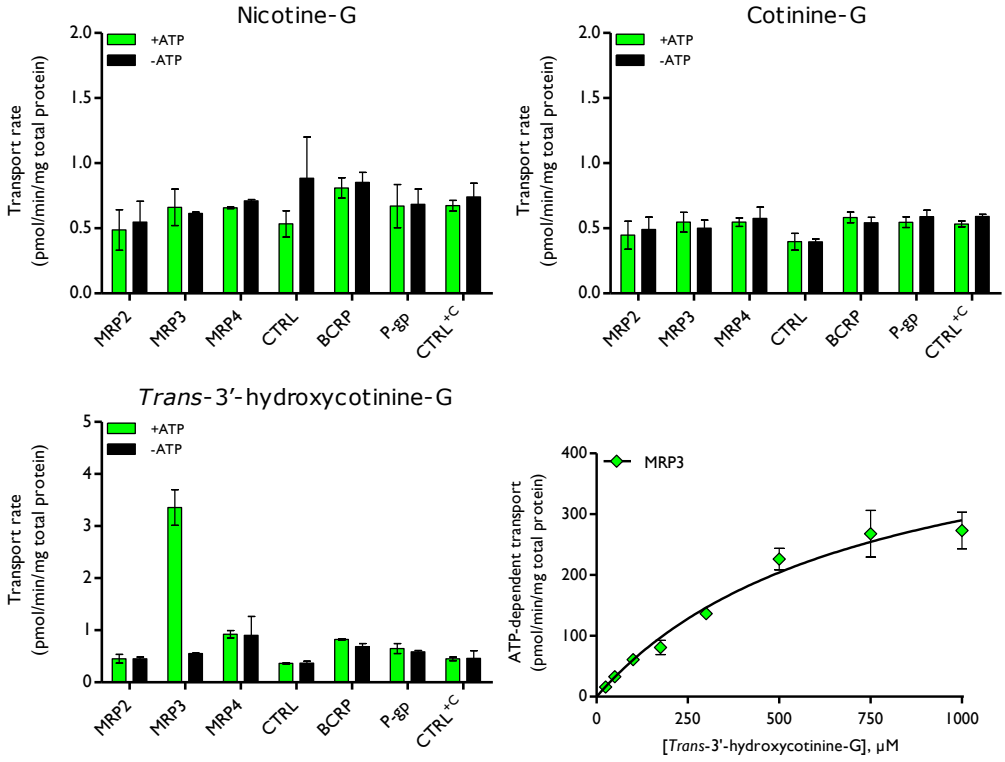


Figure 10 *Single concentration transport of nicotine-G, cotinine-G and trans-3'hydroxycotinine-G, and MRP3 transport kinetics of trans-3'-hydroxycotinine-G. The single concentration transport reactions included 5 µM of the compound. The transport reactions were incubated for 10 min. The incubation time in MRP3 transport kinetic assay was 10 min, and the solid line represents fitting of the data to the Michaelis-Menten equation.*

5.4 Transport of estrogen glucuronides (III)

BCRP was highly active in the transport of estrone-3-G and estradiol-3-G, while substantially weaker transport activity of BCRP was found toward both estriol glucuronides (Figure 11). Interestingly, the BCRP-mediated transport activity increased in the same order as the $logD_{7.4}$ values of the estrogen glucuronides investigated, which are -2.70, -2.17, -1.63 and -1.06 for estriol-16α-G, estriol-3-G, estradiol-3-G and estrone-3-G, respectively (Table 6). MRP2 and MRP3 transported all the estrogen glucuronides tested, while MRP4 was clearly active only toward estriol-16α-G (Figure 11). The transport activity of P-gp was similar to the control vesicles, $CTRL^{+C}$.

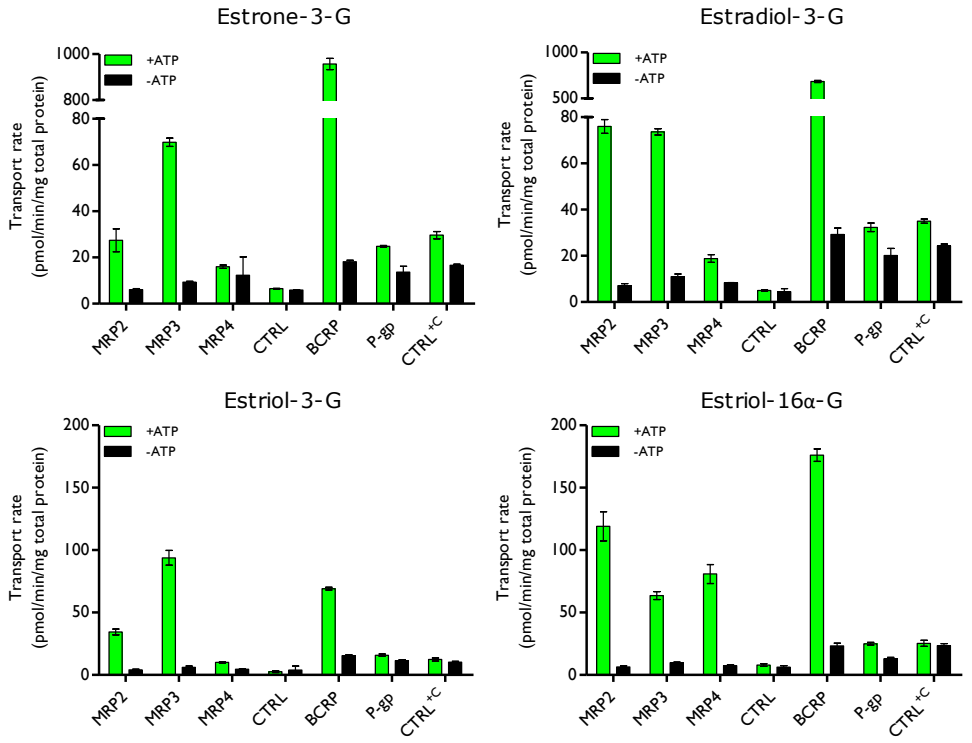


Figure 11 *Single concentration transport of the estrogen glucuronides. The concentration was 10 µM in the assays that were incubated for 4 min.*

The kinetic analyses revealed clear differences between MRP2 and MRP3 in the transport of all four estrogen glucuronides (Figure 12). The MRP3-mediated transport saturated at low concentrations, which resulted in K_m values below 20 µM, whereas the MRP2-mediated transport saturated at higher concentrations and resulted in K_m values between 200 and 800 µM (Table 9). Similar to the single concentration transport assays (Figure 11), BCRP transported estrone-3-G and estradiol-3-G at high V_{max} values in comparison to estriol glucuronides (Figure 12 and Table 9). Finally, the MRP4-mediated transport of estriol-16α-G resulted in a K_m value of 65 µM, which is over 10-fold higher than the corresponding value for MRP3 but over 10-fold lower in comparison to MRP2.

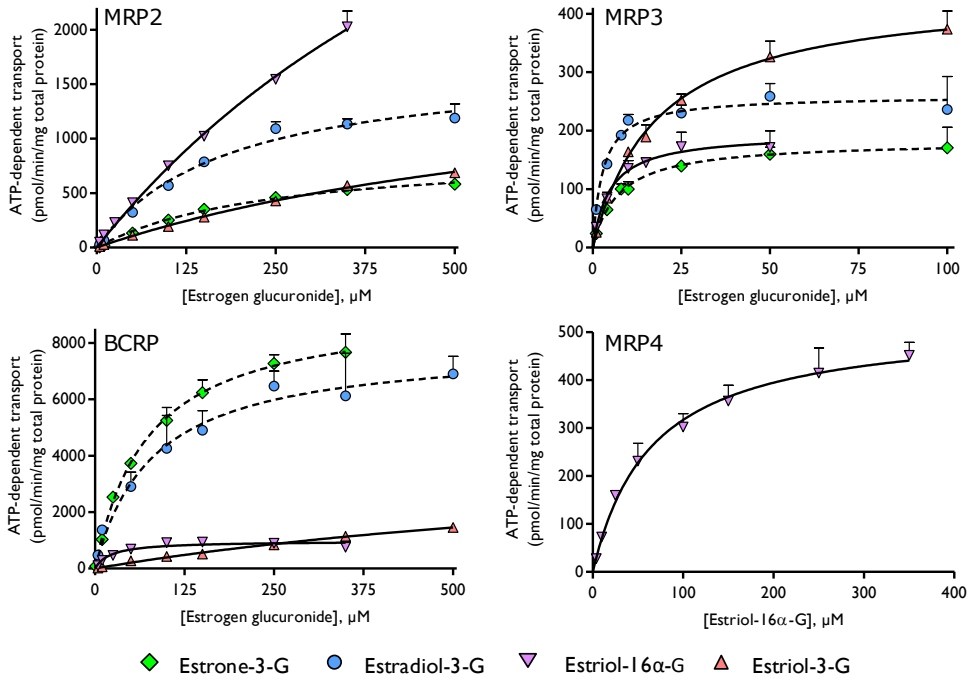


Figure 12 *The transport kinetics of the estrogen glucuronides. The incubation time in the transport reactions was 2 min, except 1 min for the MRP3-mediated transport of estradiol-3-G, and 6 min for MRP2-mediated transport of estrone-3-G and estriol-3-G, as well as BCRP-mediated transport of estriol-3-G. The lines represent fitting of the data to the Michaelis-Menten equation. Only error bars above data points are shown for clarity.*

Table 9. *The derived kinetic constants for the transport kinetic data in Figure 12.*

Substrate	K_m (95% CI), µM	V_{max} (95% CI), pmol/min/mg total protein
	MRP2	
Estrone-3-G	241 (198-285)	884 (810-960)
Estradiol-3-G	180 (130-230)	1700 (1510-1900)
Estriol-3-G	791 (585-997)	1800 (1470-2120)
Estriol-16α-G	773 (532-1010)	6440 (4930-7940)
	MRP3	
Estrone-3-G	7.28 (5.06-9.50)	182 (167-198)
Estradiol-3-G	2.83 (1.76-3.91)	260 (240-280)
Estriol-3-G	18.2 (14.7-21.6)	441 (411-471)
Estriol-16α-G	4.82 (2.82-6.82)	195 (174-216)
	BCRP	
Estrone-3-G	73.8 (62.1-85.5)	9310 (8810-9810)
Estradiol-3-G	80.9 (39-123)	7910 (6700-9130)
Estriol-3-G	1020 (621-1418)	4410 (3140-5690)
Estriol-16α-G	22.4 (11.1-33.6)	974 (861-1090)
	MRP4	
Estriol-16α-G	64.8 (48.4-81.2)	522 (479-565)

5.5 Transport of androgen glucuronides (IV)

Neither BCRP, MRP4 nor P-gp transported any of the five androgen glucuronides tested, whereas MRP2 and MRP3 were active toward them, even though at variable rates (Figure 13). MRP3 was clearly more active than MRP2 in the transport of androsterone-G, epitestosterone-G and etiocholanolone-G (Figure 13). All these structures have their glucuronic acid pointing to the opposite direction than the methyl groups of the androgen scaffold (Table 6). On the other hand, the activities of MRP2 and MRP3 in the transport of dehydroepiandrosterone-G and testosterone-G were similar.

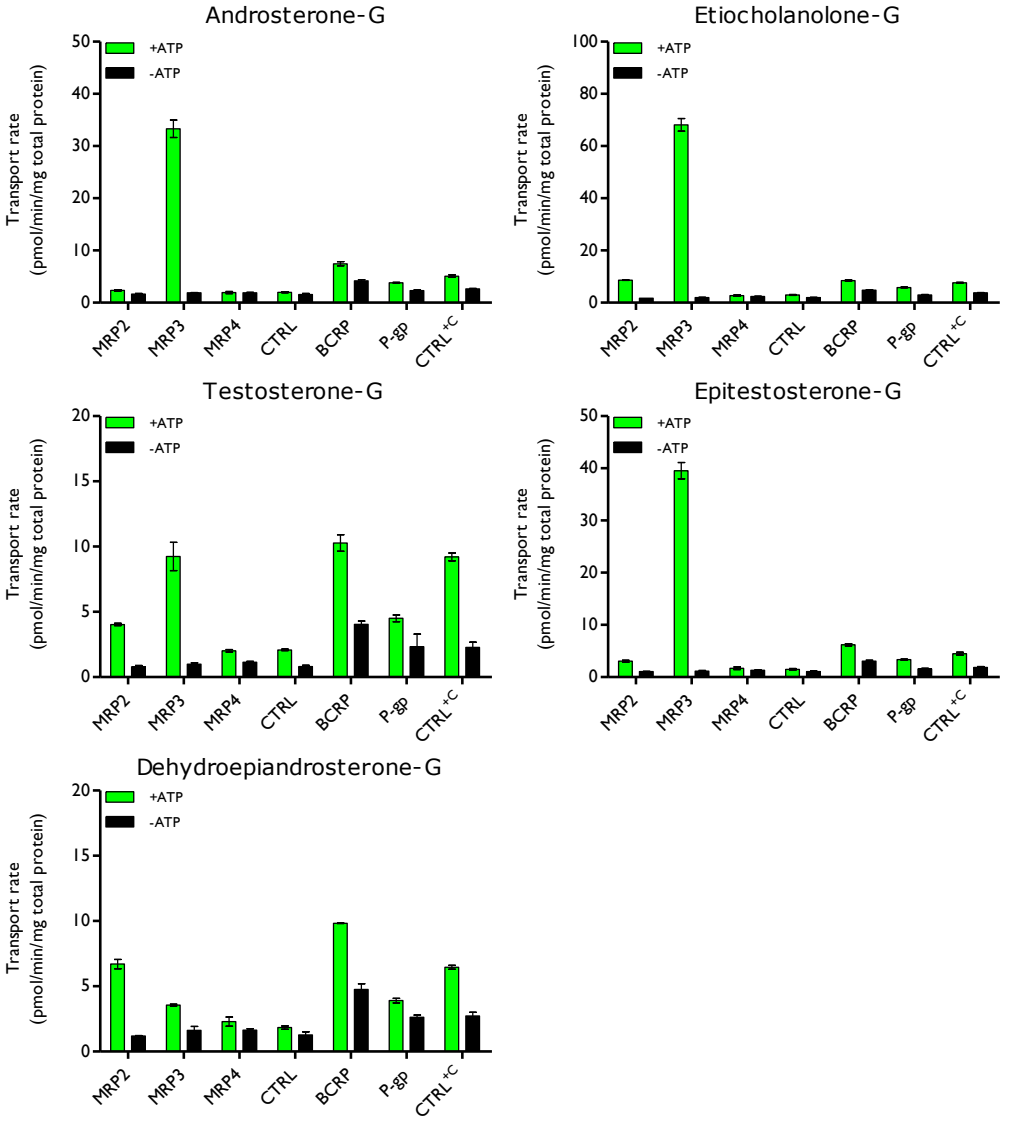


Figure 13 *The single concentration transport assays of the androgen glucuronides. The concentration was 1 µM and the incubation time was 3 min.*

The transport kinetic characterization of MRP2 and MRP3 revealed that the K_m values of MRP2 for androgen glucuronides were much higher than the respective values of MRP3 (Figure 14 and Table 10). This resembles the case of estrogen glucuronides in which the similar differences between these two transporters were found (Figure 12 and Table 9). Interestingly, the MRP2 transport kinetics of androsterone-G and epitestosterone-G were fitted better to Hill's equation than to the Michaelis-Menten equation (Table 10), although the resulting *h*-values were lower (1.4) than in the case of estradiol-17β-G (h = 2.3, Table 7). Furthermore, the apparent affinity of androgen glucuronides to MRP3 ranged from the K_m value of 0.36 μM for etiocholanolone-G to the K_m value of 51 μM for dehydroepiandrosterone-G. The kinetic constants of MRP2 transport of different androgen glucuronides were at similar range and these high K_m values suggest a low affinity transport of androgen glucuronides by MRP2 (Table 10).

Table 10. *The derived kinetic constants for the transport kinetic data in Figure 14.*

Substrate	K_m (95% CI), μM	V_{max} (95% CI), pmol/min/mg total protein
	MRP2	
Androsterone-G	S_{50} = 160 (114-206) h = 1.41 (1.10-1.72)	344 (290-399)
Dehydroepiandrosterone-G	155 (130-180)	816 (761-871)
Epitestosterone-G	S_{50} = 197 (107-287) h = 1.44 (0.98-1.90)	484 (353-614)
Etiocholanolone-G	120 (57.7-183)	692 (555-828)
Testosterone-G	428 (310-546)	1410 (1180-11640)
	MRP3	
Androsterone-G	3.65 (2.99-4.32)	295 (281-310)
Dehydroepiandrosterone-G	51.0 (39.1-62.9)	88.4 (78-98.8)
Epitestosterone-G	1.93 (1.33-2.52)	202 (189-216)
Etiocholanolone-G	0.357 (0.188-0.527)	203 (193-212)
Testosterone-G	13.7 (7.14-20.3)	136 (111-161)

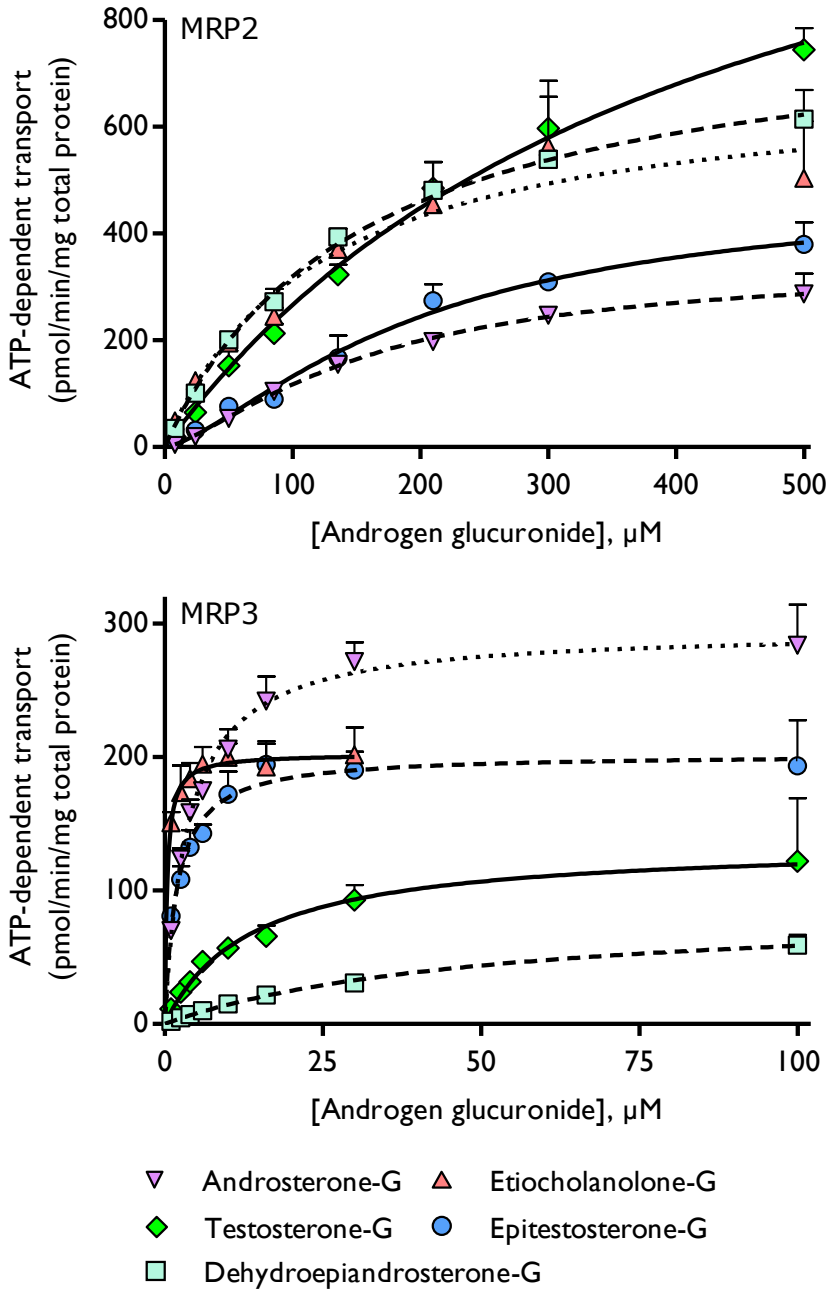


Figure 14 *The transport kinetics of the androgen glucuronides. The incubation time in the transport reactions was 1 min for MRP3, except 3 min in the case of dehydroepiandrosterone-G. Transport reaction incubation times for MRP2 were 2 and 6 min in the case of epitestosterone-G and androsterone-G, respectively, and 4 min in the case of the other glucuronides. The lines represent fitting of the data to the Michaelis-Menten equation, except for MRP2 and its transport of androsterone-G and epitestosterone-G that were fitted to Hill's equation. Only error bars above data points are shown for clarity.*

6 DISCUSSION

6.1 Structural features in the substrates of MRP2, MRP3, MRP4 and BCRP

MRP3 transported every glucuronide that was investigated in this thesis, with the exception of nicotine-G and cotinine-G. It is difficult to explain why MRP3, or any other transporter, was unable to transport these two glucuronides. One possible reason can be the positively charged nitrogen atoms in nicotine-G and cotinine-G, which are otherwise rather similar structures to the other glucuronides investigated in this thesis (Table 6). MRPs transport mostly anionic compounds, and thus it is possible that these transporters repel structures with positive charges.[85] Apart from these two compounds, MRP3 appears to be a highly versatile transporter for all types of glucuronides, even if the apparent affinities may greatly vary between different compounds. MRP3 transports several glucuronides at low K_m values, ranging from 0.2 to 14 μM, including the glucuronides of ethinylestradiol, etoposide, hyocholate, hyodeoxycholate, resveratrol and several phytoestrogens, including enterodiol and genistein.[177,191,198-200] On the other hand, the uptake of morphine-3-glucuronide into MRP3 vesicles is high but occurs at a low affinity with a K_m value over 500 μM.[173,174] Both the aforementioned reports are in line with the results in this thesis, which indicate that MRP3 is a high affinity glucuronide transporter, particularly in the case of steroid glucuronides, but in some cases it might exhibit high K_m values toward its substrates. The presence of a basic nitrogen in the structure is a common feature shared by the low affinity substrates of MRP3, among which are morphine-3-glucuronide ($logD_{7.4}$ = -3.49, PSA = 149 Å^2), *trans*-3'-hydroxycotinine-G, R- and S-propranolol-G. As discussed above, this feature might hamper the transport of MRP3.

MRP2 appears to be a more selective transporter, with lower affinities toward the glucuronides investigated in this thesis. Some smaller glucuronides are not transported by MRP2 (I and II), whereas the steroid glucuronides are substrates of MRP2 with high V_{max} but also high K_m values (III and IV). These results indicate that the binding site of MRP2 might be larger and more flexible in comparison to MRP3. This is further supported by the findings that MRP2, but not MRP3, transports glutathione conjugates that are rather large due to the high MW of the glutathione moiety, 307 Da.[125,201] Furthermore, Kato and coworkers analyzed the uptake of 10 different β-lactam antibiotics into human MRP2 vesicles and found that only compounds with a MW over 500 Da were transported efficiently.[202] In addition, MRP2 does not transport the glucuronide conjugates of 7-hydroxycoumarin, edaravone and gaboxadol, all of which have a MW below 350 Da.[141,143,145] Together, MRP2 and MRP3 transport a wide range of glucuronides, but MRP2 appears to be restricted to higher MW glucuronides and have high K_m values, whereas MRP3 mostly exhibits a high affinity transport of the same compounds (Table 6 and the Results section).

The findings on MRP4 within this thesis are interesting, because this transporter was active in the transport of only five of the compounds investigated. The intrinsic molecular properties of these compounds (1-hydroxypyrene-G, 1-naphthol-G, 4-methylumbelliferone-G, estradiol-17β-G and estriol-16α-G) are different and the reasons for the substrate selectivity of MRP4 are difficult to explain. In particular, because the K_m values differed from low to moderate, 3 to 170 μM. MRP4 efficiently transports glucuronides of edaravone and 7-hydroxycoumarin, with the K_m values of 10 and 60 μM, respectively.[143,145] These values are in a similar range with the MRP4 substrates identified in this thesis (Table 7-9). In addition, MRP4 transports estradiol-17β-G and gemfibrozil

glucuronide similarly to MRP2 and MRP3, but it does not transport the glucuronides of E3040, morphine or troglitazone even though these compounds are substrates for MRP2 and MRP3.[158,174] Furthermore, MRP4 is able to transport a variety of different compounds such as cAMP, cGMP, dehydroepiandrosterone sulfate, as well as prostaglandin E1 and E2.[195,203,204] Thus, MRP4 is a more selective transporter than MRP2 and MRP3, and among the substrates and non-substrates identified in this thesis it is difficult to define the reasons for MRP4's selectivity (Table 6).

Multiple flavonoid glucuronides may be transported by the human BCRP according to data from in vivo Bcrp-knockout mice studies (Table 5). In vitro studies with human BCRP membrane vesicles have confirmed the high transport of several glucuronide conjugates of flavonoids, including the glucuronides of baicalein, scutellarein, resveratrol and different dihydroxyflavonones.[142,177,205,206] These flavonoid glucuronides share a rather flat structure that contains two or three aromatic rings. Furthermore, BCRP efficiently transports the glucuronide conjugate of SN-38, an active metabolite of the anticancer drug irinotecan, with a K_m value of approximately 20 μM.[207] The structure of SN-38 is completely planar, with the exception of two ethyl side chains, and it contains aromatic rings. The results from this thesis further support the role of BCRP in the disposition of glucuronide conjugates, although the BCRP-mediated transport appears to be rather selective for flat and aromatic structures. A recently solved high-resolution cryo-electron microscopy structure of BCRP revealed a slit substrate-binding pocket, which is able to accommodate flat, hydrophobic and polycyclic substrates.[208]

BCRP has been postulated to be more important in the transport of sulfate conjugates than glucuronides.[209-211] Although animal Bcrp-knockout studies have revealed that BCRP is capable of transporting glucuronide conjugates as well (Table 5), data with human transporters are still scarce.[125] This thesis deepens the understanding of the significance of BCRP in the transport of glucuronide conjugates. First, BCRP appears to be more selective by transporting fewer glucuronides than MRP2 and MRP3, as discussed above. However, in some cases BCRP should be considered as the transporter that affects biliary and intestinal excretion of glucuronide metabolites. Second, BCRP might be a low affinity but high capacity transporter for glucuronides in comparison to sulfate conjugates. For example, the K_m value for estrone-3-G (74 μM) is over a magnitude higher than for estrone sulfate (1 μM) but the V_{max} for estrone sulfate is 10-fold lower (III), which results in only 5-fold higher intrinsic clearance for the sulfate conjugate by BCRP (III). Similarly, BCRP transports resveratrol sulfate at a higher apparent affinity than the corresponding glucuronide, the K_m values of 5 versus 120 μM.[177] In addition, 4-methylumbelliferone-G is a weak substrate for BCRP (I), while the sulfate conjugate of the same compound is highly transported by BCRP.[210]

None of the glucuronides tested had higher uptake into P-gp vesicles in comparison to the control vesicles. The data in this thesis further verify that P-gp is not involved in the transport of glucuronide metabolites of drugs.[125] Furthermore, the P-gp transport results in this thesis emphasize the importance of proper control preparations within in vitro assays, because some of the compounds studied were transported into P-gp vesicles but similar transport occurred into the corresponding control vesicles ($CTRL^{+C}$). For example, estradiol-17β-G is a rare exception among glucuronides reported as substrates for P-gp in the vesicular transport assay.[212] However, Huang and coworkers employed non-infected control cells for the control vesicle preparations, which likely exhibited a lower expression of endogenous transporters and subsequently a lower transport activity.[212] In this thesis, the control vesicles were prepared either from the empty-virus infected cells (I and III) or from cells that were infected with a virus encoding a transport-deficient protein of a human transporter (II and IV). As a result, the control preparations used in this thesis have similar endogenous transport activity as cells infected with the virus encoding a functional human

recombinant transporter. Furthermore, cholesterol addition to the membrane vesicle preparations is crucial for the proper transport activity of BCRP and P-gp, but this addition also activates endogenous transporters.[192,193,213] Due to this high activity in the control preparations, determination of transport kinetics for the BCRP-mediated transport of 4-methylumbelliferone-G and 1-naphthol-G was not feasible.

6.2 Impact of the efflux transporters on glucuronide metabolite disposition

Important issues that remain to be solved are the tissue-specific function of BCRP and in which tissues this transporter affects the elimination of drug metabolites. Undeniably, BCRP is markedly important in restricting the entry of drugs to the brain over the BBB, together with P-gp.[7,70] In addition, a few but significant BCRP-mediated drug-drug interactions are due to the inhibition of intestinal BCRP, which supports a role for BCRP in the absorption of drugs.[214] Nevertheless, the expression of this transporter in the human liver is 10-fold lower than the expression of MRP2, and some studies even suggest that BCRP is almost absent from the human liver.[77,83] Similarly, the protein expression of BCRP in the human kidney is low or has not been detected.[86-88] Unfortunately, no comprehensive data are available for the hepatic expression of Bcrp in mice, but it is suggested to be low, as in humans, whereas the expression of this transporter in the mouse kidney is much higher in comparison to the human tissue.[86,215]

Based on the results from animal experiments, Bcrp affects the systemic exposure and biliary excretion of several glucuronide and sulfate metabolites after either oral or parenteral administration of the parent compound (Table 5). For example, the cumulative biliary excretion of 4-methylumbelliferone-G was reduced to half in liver perfusions from both Mrp2- and Bcrp-knockout mice. On the other hand, only the knockout of Bcrp, but not Mrp2, affected the biliary excretion of 4-methylumbelliferone sulfate by abolishing its excretion almost completely.[148] Furthermore, the CL_r of 4-methylumbelliferone sulfate was unchanged in Bcrp-knockout mice after intravenous infusion of it, although the same strain of mice exhibited decreased CL_r of E3040 sulfate by two-fold in comparison to wild type animals.[216] Both 4-methylumbelliferone and E3040 sulfate are actively secreted in urine in mice and are highly transported by the human BCRP.[210,216] These contradictory findings could be partly explained by the different f_e values of Bcrp in different tissues and for different compounds. First, in the mouse livers, the f_e for the Bcrp-mediated biliary excretion of 4-methylumbelliferone sulfate is 1.[180] Second, the respective f_e values for Mrp2 and BCRP in the case of 4-methylumbelliferone-G are 0.33 and 0.73, respectively. Therefore, the sole contribution of Bcrp to the biliary excretion of 4-methylumbelliferone sulfate explains the complete abolishment of its biliary excretion in the Bcrp-knockout mice. On the other hand, the mild effect of Bcrp-knockout on the biliary excretion of 4-methylumbelliferone-G is explained by similar contribution of both Mrp2 and Bcrp to this excretion pathway (Section 2.3.2 and Equation 5). Lastly, Mrp4 contributes to the basolateral hepatic excretion of 4-methylumbelliferone sulfate.[149] The activity of Mrp4 may compensate for the impaired renal excretion of 4-methylumbelliferone sulfate also in the kidneys of the Bcrp-knockout mice and, therefore, explain the unchanged CL_r of this compound in the knockout mice in comparison to wild type mice. Thus, not only the absolute protein expression of a transporter in a tissue affects its contribution to the excretion of its substrates, but also the relative contribution of the transporter in each tissue is an important parameter.

Estrone-3-G and estradiol-3-G were identified as the best substrates for BCRP within this thesis, but none of the androgen glucuronides were transported by this transporter. Apart from dehydroepiandrosterone-G, all the estrogen and androgen glucuronides were transported by MRP3 at high apparent affinities. Furthermore, MRP2 contributed to the transport of the same steroid glucuronides, even if at high K_m values. Estrone and estradiol are excreted about 50% to bile in humans after parenteral administration, whereas the biliary excretion of estriol is around 20%.[217,218] Moreover, the biliary excretion of testosterone is about 10% in humans, whereas for androsterone and etiocholanolone it is negligible.[219,220] Since these steroids are excreted as conjugates, BCRP may play a key role in the high biliary excretion of estrogens, but not androgens.

MRP2 and MRP3 did not appear to distinguish between androgen and estrogen glucuronides but transported both type of compounds. Thus, it is evident that these conjugates are excreted via both bile and urine. However, the relative contribution of these two pathways is difficult to estimate. It is interesting to speculate whether the lower affinity of testosterone-G to MRP3 in comparison to androsterone-G and etiocholanolone-G increases the relative contribution of MRP2 to the biliary excretion of testosterone-G. On the other hand, the contribution of hepatic uptake transporters, particularly OATPs, to the disposition of androgen glucuronides cannot be excluded. Estradiol-17β-G is a well-established substrate for the human hepatic uptake transporters OATP1B1 and OATP1B3, which can reduce the urinary excretion of this glucuronide by the so-called hepatocyte hopping.[68,221] This phenomenon enhances the transport of the glucuronide back into hepatocytes from the basolateral side and its subsequent availability for the biliary excretion, even in the presence of high basolateral efflux. Unfortunately, no in vitro data on human hepatic uptake of androgen glucuronides or other estrogen glucuronides are currently available. However, data are available on human in vivo excretion of several androgen and estrogen glucuronides following their parenteral administration. In humans, about 50% and 7% of parenterally administered estradiol-17β-G and estrone-3-G are excreted in bile, whereas about 20% of estriol-16α-G is excreted via bile.[222,223] In contrast, estriol-3-G or androsterone-G are not excreted in bile but are rapidly cleared in urine after their parenteral administration.[224,225] Furthermore, estradiol-3-G and testosterone-G partly undergo metabolism after their parenteral administration suggesting active transport into hepatocytes and excretion in bile, although this pathway accounts for less than 50% of the dose based on the formed metabolites.[226,227] Thus, it is clear that even in the presence of high basolateral efflux of a metabolite from the liver, the sum of influx and efflux over the basolateral membrane, namely the net flux, determines the disposition of a metabolite and be the driving force for biliary excretion.

The glucuronides of 4-methylumbelliferone, R- and S-propranolol and *trans*-3'-hydroxycotinine were not substrates for MRP2 or BCRP but were transported by MRP3, or both MRP3 and MRP4 in the case of 4-methylumbelliferone-G. In humans, the clearance of 4-methylumbelliferone via glucuronidation is high, and the rapid urinary excretion of 4-methylumbelliferone-G accounts for 93% of the total dose.[228] Propranolol glucuronides and *trans*-3'-hydroxycotinine-G are also completely excreted in urine.[229-232] Furthermore, 4-methylumbelliferone-G is neither a substrate for OATP1B1 nor actively transported into rat hepatocytes.[121,158] Similar data are not available for propranolol glucuronides, but these metabolites are accumulated almost 20-fold in blood of patients with renal failure, which suggests no major hepatic uptake for propranolol glucuronides, but rather excessive renal excretion.[233] Hence, MRP3 is probably, and almost solely, responsible for the hepatic excretion of 4-methylumbelliferone-G, *trans*-3'-hydroxycotinine-G, as well as R- and S-propranolol-G. On the other hand, the contribution of hepatic MRP4 to the transport of 4-methylumbelliferone-G is

difficult to estimate, because the expression level of this transporter in the human liver is generally low compared to that of MRP3.[83]

In the kidney, MRP4 appears to be an important efflux transporter for the active secretion of glucuronides, which is suggested by the high CL_r of 4-methylumbelliferone-G and estriol-16α-G, exceeding the GFR by at least three-fold.[228,234] On the other hand, the CL_r of androsterone-G, etiocholanolone-G, estriol-3-G and propranolol glucuronides are similar to the GFR, and no active secretion for *trans*-3'-hydroxycotinine-G has been reported.[230,234-236] Thus, MRP4 likely contributes to the active renal secretion of 4-methylumbelliferone-G and estriol-16α-G but not to that of propranolol glucuronides, estriol-3-G or *trans*-3'-hydroxycotinine-G. These findings agree well with the in vitro results of this thesis, according to which only the former two compounds are substrates for MRP4. It should be noted that the glucuronidation of 4-methylumbelliferone and estriol in the kidney could not be excluded, because the UGTs that catalyze their glucuronidation are expressed also in this tissue.[31,237,238]

6.3 Future prospects

MRP2 and MRP3 appear to be rather unselective in the transport of different glucuronides, and particularly MRP3 transported all the glucuronides investigated, except nicotine-G and cotinine-G. The transport of these two glucuronides was also tested in MRP1, MRP5 and MRP6 membrane vesicles, but without success (II). Interestingly, similar examples have been recently reported where no currently known transporter could be identified for the active uptake or efflux of a drug.[239,240] For example, atovaquone is an anionic drug that is exceptionally highly concentrated in human bile.[95] Patel and coworkers found in vitro biliary excretion of atovaquone in human hepatocytes, however, they could not identify neither the uptake nor the efflux transporter among the known drug transporters for this drug.[240] Thus, it is possible that several other transporters that affect drug and drug metabolite disposition are still to be discovered.

The empirical MW threshold value for biliary excretion of anionic compounds is about 400-500 Da.[28,100,101] In addition, the ECCS predicts that acids with MW above 400 Da are primarily cleared from the systemic circulation by hepatic OATPs.[6] Even if these values can only be used as good estimators for the main excretion route of a drug or drug metabolite, some correlation appears to be found within the glucuronides investigated in this thesis and their biliary excretion. For example, the glucuronides below 400 Da were not substrates for MRP2 and many of them are excreted exclusively in urine. On the other hand, literature reports suggest that some of the glucuronides above 400 Da included in this thesis may be substrates for the hepatic uptake transporters but with rather different efficiencies, as discussed above. Hence, it would be essential and interesting, in the future, to understand better the substrate specificities of hepatic OATPs and their effects on the net flux over the hepatic basolateral membrane. Specific questions would be whether the substrate preferences of OATPs and MRP2 significantly overlap and whether the OATP-MRP2 interplay determines the empirical MW threshold value of 400-500 Da for anionic compounds, such as glucuronides.

In addition to OATPs, OAT2 and OAT7 in the liver as well as OAT1 and OAT3 in the kidney may affect the disposition of glucuronide metabolites.[7,73,74] So far, no well-characterized glucuronide conjugate substrates have been reported for OAT2 or OAT7.[74,105,123,241,242] On the other hand, several glucuronides have been reported as substrates for OAT3, and a few for OAT1.[105,123,183,184,242-244] Thus, it would be interesting to study the OAT1/3-MRP4 interplay in the

kidney in more detail and whether 4-methylumbelliferone-G or estriol-16-G are substrates also for the renal OATs. In addition, MRP2 is expressed in the same membrane with MRP4 in the kidney and it transports androsterone-G, etiocholanolone-G and estriol-3-G, but the CL_r of these metabolites is similar to the GFR.[234,235] Hence, it could be suggested that they are not substrates for OAT1 or OAT3, and that the overlapping substrate preference exists only between OAT1/3 and MRP4.

7 Conclusions

Efflux transport of several glucuronide metabolites of drugs and drug-like compounds, such as androgens and estrogens, were investigated in this thesis, employing the vesicular transport assay. The human efflux transporters MRP2, MRP3, MRP4, BCRP and P-gp were included in the studies. Based on the results, MRP2 and MRP3 are rather versatile transporters that transport almost all the compounds investigated, whereas MRP4 and BCRP appeared to be more selective and transport only some of the compounds. Transport kinetic analyses revealed mostly low K_m values for MRP3 and the lowest value was even below 1 μM. On the other hand, MRP2 had clearly lower apparent affinities, K_m values over 100 μM, or even no transport of some substrates that MRP3 transported. MRP4 transported only one of the nine steroid glucuronides investigated, but had high activity toward glucuronides containing small planar rings. BCRP was highly active only toward estrogen glucuronides and 1-hydroxypyrene glucuronide, which indicates that aromatic rings are important for binding to this transporter. P-gp did not transport any of the glucuronides investigated, and therefore it appears that this transporter does not contribute to the disposition of glucuronide metabolites of drugs in humans.

Together, MRP2 and MRP3 are important efflux transporters in the human liver and intestine, where they affect the disposition of a high variety of different glucuronide metabolites of drugs. BCRP and MRP4 may be important in specific cases, MRP4 particularly in the kidney where it contributes to the active secretion of some glucuronide metabolites.

References

1. Di, L. The role of drug metabolizing enzymes in clearance. *Expert Opin. Drug Metab. Toxicol.* **2014**, *10*, 379-393.
2. Cerny, M. A. Prevalence of non-cytochrome P450-mediated metabolism in food and drug administration-approved oral and intravenous drugs: 2006-2015. *Drug Metab. Dispos.* **2016**, *44*, 1246-1252.
3. Testa, B.; Pedretti, A.; Vistoli, G. Reactions and enzymes in the metabolism of drugs and other xenobiotics. *Drug Discov. Today* **2012**, *17*, 549-560.
4. Döring, B.; Petzinger, E. Phase 0 and phase III transport in various organs: Combined concept of phases in xenobiotic transport and metabolism. *Drug Metab. Rev.* **2014**, *46*, 261-282.
5. Smith, D. A.; Beaumont, K.; Maurer, T. S.; Di, L. Clearance in Drug Design. *J. Med. Chem.* **2019**, *62*, 2245-2255.
6. Varma, M. V.; Steyn, S. J.; Allerton, C.; El-Kattan, A. F. Predicting Clearance Mechanism in Drug Discovery: Extended Clearance Classification System (ECCS). *Pharm. Res.* **2015**, *32*, 3785-3802.
7. Giacomini, K. M.; Huang, S. -.; Tweedie, D. J.; Benet, L. Z.; Brouwer, K. L. R.; Chu, X.; Dahlin, A.; Evers, R.; Fischer, V.; Hillgren, K. M.; et al. Membrane transporters in drug development. *Nat. Rev. Drug Discov.* **2010**, *9*, 215-236.
8. Rautio, J.; Meanwell, N. A.; Di, L.; Hageman, M. J. The expanding role of prodrugs in contemporary drug design and development. *Nat. Rev. Drug Discov.* **2018**, *17*, 559-587.
9. Varma, M. V. S.; Obach, R. S.; Rotter, C.; Miller, H. R.; Chang, G.; Steyn, S. J.; El-Kattan, A.; Troutman, M. D. Physicochemical space for optimum oral bioavailability: Contribution of human intestinal absorption and first-pass elimination. *J. Med. Chem.* **2010**, *53*, 1098-1108.
10. Shitara, Y.; Horie, T.; Sugiyama, Y. Transporters as a determinant of drug clearance and tissue distribution. *Eur. J. Pharm. Sci.* **2006**, *27*, 425-446.
11. Williams, J. A.; Hyland, R.; Jones, B. C.; Smith, D. A.; Hurst, S.; Goosen, T. C.; Peterkin, V.; Koup, J. R.; Ball, S. E. Drug-drug interactions for UDP-glucuronosyltransferase substrates: A pharmacokinetic explanation for typically observed low exposure (AUC 1/AUC) ratios. *Drug Metab. Dispos.* **2004**, *32*, 1201-1208.
12. Guillemette, C.; Lévesque, É; Rouleau, M. Pharmacogenomics of human uridine diphospho-glucuronosyltransferases and clinical implications. *Clin. Pharmacol. Ther.* **2014**, *96*, 324-339.
13. Guengerich, F. P. Common and uncommon cytochrome P450 reactions related to metabolism and chemical toxicity. *Chem. Res. Toxicol.* **2001**, *14*, 611-650.
14. Rendic, S.; Guengerich, F. P. Survey of human oxidoreductases and cytochrome P450 enzymes involved in the metabolism of xenobiotic and natural chemicals. *Chem. Res. Toxicol.* **2015**, *28*, 38-42.
15. Zanger, U. M.; Schwab, M. Cytochrome P450 enzymes in drug metabolism: Regulation of gene expression, enzyme activities, and impact of genetic variation. *Pharmacol. Ther.* **2013**, *138*, 103-141.
16. Yu, J.; Zhou, Z.; Tay-Sontheimer, J.; Levy, R. H.; Ragueneau-Majlessi, I. Risk of clinically relevant pharmacokinetic-based drug-drug interactions with drugs approved by the U.S. Food and drug administration between 2013 and 2016. *Drug Metab. Dispos.* **2018**, *46*, 835-845.
17. Yu, J.; Petrie, I. D.; Levy, R. H.; Ragueneau-Majlessi, I. Mechanisms and clinical significance of pharmacokinetic-based drug-drug interactions with drugs approved by the U.S. Food and drug administration in 2017 s. *Drug Metab. Dispos.* **2019**, *47*, 135-144.
18. Fukami, T.; Yokoi, T. The emerging role of human esterases. *Drug Metab. Pharmacokinet.* **2012**, *27*, 466-477.
19. Oda, S.; Fukami, T.; Yokoi, T.; Nakajima, M. A comprehensive review of UDP-glucuronosyltransferase and esterases for drug development. *Drug Metab. Pharmacokinet.* **2015**, *30*, 30-51.
20. Rowland, A.; Miners, J. O.; Mackenzie, P. I. The UDP-glucuronosyltransferases: Their role in drug metabolism and detoxification. *Int. J. Biochem. Cell Biol.* **2013**, *45*, 1121-1132.

21. Stingl, J. C.; Bartels, H.; Viviani, R.; Lehmann, M. L.; Brockmöller, J. Relevance of UDP-glucuronosyltransferase polymorphisms for drug dosing: A quantitative systematic review. *Pharmacol. Ther.* **2014***, 141*, 92-116.
22. Benedetti, M. S.; Whomsley, R.; Baltes, E. Involvement of enzymes other than CYPs in the oxidative metabolism of xenobiotics. *Expert Opin. Drug Metab. Toxicol.* **2006***, 2*, 895-921.
23. Hayes, J. D.; Flanagan, J. U.; Jowsey, I. R. Glutathione transferases. *Annu. Rev. Pharmacol. Toxicol.* **2005***, 45*, 51-88.
24. Lo, W. -.; Chiou, Y. -.; Hsu, Y. -.; Lam, W. S.; Chang, M. -.; Jao, S. -.; Li, W. -. Enzymatic and nonenzymatic synthesis of glutathione conjugates: Application to the understanding of a parasite's defense system and alternative to the discovery of potent glutathione S-transferase inhibitors. *Bioconjugate Chem.* **2007***, 18*, 109-120.
25. Rendic, S.; Guengerich, F. P. Contributions of human enzymes in carcinogen metabolism. *Chem. Res. Toxicol.* **2012***, 25*, 1316-1383.
26. Zimmermann, M.; Zimmermann-Kogadeeva, M.; Wegmann, R.; Goodman, A. L. Mapping human microbiome drug metabolism by gut bacteria and their genes. *Nature* **2019***, 570*, 462-467.
27. Pollet, R. M.; D'Agostino, E. H.; Walton, W. G.; Xu, Y.; Little, M. S.; Biernat, K. A.; Pellock, S. J.; Patterson, L. M.; Creekmore, B. C.; Isenberg, H. N.; et al.. An Atlas of ß-Glucuronidases in the Human Intestinal Microbiome. *Structure* **2017***, 25*, 967-977.e5.
28. Roberts, M. S.; Magnusson, B. M.; Burczynski, F. J.; Weiss, M. Enterohepatic circulation: Physiological, pharmacokinetic and clinical implications. *Clin. Pharmacokinet.* **2002***, 41*, 751-790.
29. Cubitt, H. E.; Houston, J. B.; Galetin, A. Prediction of human drug clearance by multiple metabolic pathways: Integration of hepatic and intestinal microsomal and cytosolic data. *Drug Metab. Dispos.* **2011***, 39*, 864-873.
30. Fallon, J. K.; Neubert, H.; Goosen, T. C.; Smith, P. C. Targeted precise quantification of 12 human recombinant uridine-diphosphate glucuronosyl transferase 1A and 2B isoforms using nano-ultra-high-performance liquid chromatography/tandem mass spectrometry with selected reaction monitoring. *Drug Metab. Dispos.* **2013***, 41*, 2076-2080.
31. Sato, Y.; Nagata, M.; Tetsuka, K.; Tamura, K.; Miyashita, A.; Kawamura, A.; Usui, T. Optimized methods for targeted peptide-based quantification of human uridine 59-diphosphate-glucuronosyltransferases in biological specimens using liquid chromatography-tandem mass spectrometry. *Drug Metab. Dispos.* **2014***, 42*, 885-889.
32. Asher, G. N.; Fallon, J. K.; Smith, P. C. UGT concentrations in human rectal tissue after multidose, oral curcumin. *Pharmacol. Res. Perspect.* **2016***, 4*.
33. Beyerle, J.; Frei, E.; Stiborova, M.; Habermann, N.; Ulrich, C. M. Biotransformation of xenobiotics in the human colon and rectum and its association with colorectal cancer. *Drug Metab. Rev.* **2015***, 47*, 199-221.
34. Drozdzik, M.; Busch, D.; Lapczuk, J.; Müller, J.; Ostrowski, M.; Kurzawski, M.; Oswald, S. Protein Abundance of Clinically Relevant Drug-Metabolizing Enzymes in the Human Liver and Intestine: A Comparative Analysis in Paired Tissue Specimens. *Clin. Pharmacol. Ther.* **2018***, 104*, 515-524.
35. Iswandana, R.; Irianti, M. I.; Oosterhuis, D.; Hofker, H. S.; Merema, M. T.; de Jager, M. H.; Mutsaers, H. A. M.; Olinga, P. Regional Differences in Human Intestinal Drug Metabolism. *Drug Metab. Dispos.* **2018***, 46*, 1879-1885.
36. Zhang, H.; Basit, A.; Busch, D.; Yabut, K.; Bhatt, D. K.; Drozdzik, M.; Ostrowski, M.; Li, A.; Collins, C.; Oswald, S.; Prasad, B. Quantitative characterization of UDP-glucuronosyltransferase 2B17 in human liver and intestine and its role in testosterone first-pass metabolism. *Biochem. Pharmacol.* **2018***, 156*, 32-42.
37. Murakami, T. Absorption sites of orally administered drugs in the small intestine. *Expert Opin. Drug. Discov.* **2017***, 12*, 1219-1232.
38. Nakamura, K.; Hirayama-Kurogi, M.; Ito, S.; Kuno, T.; Yoneyama, T.; Obuchi, W.; Terasaki, T.; Ohtsuki, S. Large-scale multiplex absolute protein quantification of drug-metabolizing

enzymes and transporters in human intestine, liver, and kidney microsomes by SWATH-MS: Comparison with MRM/SRM and HR-MRM/PRM. *Proteomics* **2016**, *16*, 2106-2117.

39. Paine, M. F.; Hart, H. L.; Ludington, S. S.; Haining, R. L.; Rettie, A. E.; Zeldin, D. C. The human intestinal cytochrome P450 "pie". *Drug Metab. Dispos.* **2006**, *34*, 880-886.
40. Gertz, M.; Houston, J. B.; Galetin, A. Physiologically based pharmacokinetic modeling of intestinal first-pass metabolism of CYP3A substrates with high intestinal extraction. *Drug Metab. Dispos.* **2011**, *39*, 1633-1642.
41. Galetin, A.; Houston, J. B. Intestinal and hepatic metabolic activity of five cytochrome P450 enzymes: Impact on prediction of first-pass metabolism. *J. Pharmacol. Exp. Ther.* **2006**, *318*, 1220-1229.
42. Hisaka, A.; Nakamura, M.; Tsukihashi, A.; Koh, S.; Suzuki, H. Assessment of intestinal availability (FG) of substrate drugs of cytochrome P450s by analyzing changes in pharmacokinetic properties caused by drug-drug interactions. *Drug Metab. Dispos.* **2014**, *42*, 1640-1645.
43. Yau, E.; Petersson, C.; Dolgos, H.; Peters, S. A. A comparative evaluation of models to predict human intestinal metabolism from nonclinical data. *Biopharm. Drug Dispos.* **2017**, *38*, 163-186.
44. Court, M. H.; Zhang, X.; Ding, X.; Yee, K. K.; Hesse, L. M.; Finel, M. Quantitative distribution of mRNAs encoding the 19 human UDP-glucuronosyltransferase enzymes in 26 adult and 3 fetal tissues. *Xenobiotica* **2012**, *42*, 266-277.
45. Nakamori, F.; Naritomi, Y.; Hosoya, K. -.; Moriguchi, H.; Tetsuka, K.; Furukawa, T.; Kadono, K.; Yamano, K.; Terashita, S.; Teramura, T. Quantitative prediction of human intestinal glucuronidation effects on intestinal availability of UDP-glucuronosyltransferase substrates using in vitro data. *Drug Metab. Dispos.* **2012**, *40*, 1771-1777.
46. Cubitt, H. E.; Houston, J. B.; Galetin, A. Relative Importance of Intestinal and Hepatic Glucuronidation-Impact on the Prediction of Drug Clearance. *Pharm. Res.* **2009**, *26*, 1073-1083.
47. Gufford, B. T.; Chen, G.; Vergara, A. G.; Lazarus, P.; Oberlies, N. H.; Paine, M. F. Milk thistle constituents inhibit raloxifene intestinal glucuronidation: A potential clinically relevant natural product-drug interaction. *Drug Metab. Dispos.* **2015**, *43*, 1353-1359.
48. Watanabe, Y.; Nakajima, M.; Yokoi, T. Troglitazone glucuronidation in human liver and intestine microsomes: High catalytic activity of UGT1A8 and UGT1A10. *Drug Metab. Dispos.* **2002**, *30*, 1462-1469.
49. Court, M. H.; Krishnaswamy, S.; Hao, Q.; Duan, S. X.; Patten, C. J.; Von Moltke, L. L.; Greenblatt, D. J. Evaluation of 3'-azido-3'-deoxythymidine, morphine, and codeine as probe substrates for udp-glucuronosyltransferase 2B7 (UGT2B7) in human liver microsomes: Specificity and influence of the UGT2B7*2 polymorphism. *Drug Metab. Dispos.* **2003**, *31*, 1125-1133.
50. Mistry, M.; Houston, J. B. Glucuronidation in vitro and in vivo. Comparison of intestinal and hepatic conjugation of morphine, naloxone, and buprenorphine. *Drug Metab. Dispos.* **1987**, *15*, 710-717.
51. Basit, A.; Amory, J. K.; Prasad, B. Effect of Dose and 5a-Reductase Inhibition on the Circulating Testosterone Metabolite Profile of Men Administered Oral Testosterone. *Clin. Transl. Sci.* **2018**, *11*, 513-522.
52. Hatfield, M. J.; Tsurkan, L.; Garrett, M.; Shaver, T. M.; Hyatt, J. L.; Edwards, C. C.; Hicks, L. D.; Potter, P. M. Organ-specific carboxylesterase profiling identifies the small intestine and kidney as major contributors of activation of the anticancer prodrug CPT-11. *Biochem. Pharmacol.* **2011**, *81*, 24-31.
53. Sato, Y.; Miyashita, A.; Iwatsubo, T.; Usui, T. Simultaneous absolute protein quantification of carboxylesterases 1 and 2 in human liver tissue fractions using liquid chromatography-tandem mass spectrometry. *Drug Metab. Dispos.* **2012**, *40*, 1389-1396.
54. Kobayashi, Y.; Fukami, T.; Nakajima, A.; Watanabe, A.; Nakajima, M.; Yokoi, T. Species differences in tissue distribution and enzyme activities of arylacetamide deacetylase in human, rat, and mouse. *Drug Metab. Dispos.* **2012**, *40*, 671-679.

55. Kurokawa, T.; Fukami, T.; Yoshida, T.; Nakajima, M. Arylacetamide Deacetylase is Responsible for Activation of Prasugrel in Human and Dog. *Drug Metab. Dispos.* **2016***, 44*, 409-416.

56. Tang, M.; Mukundan, M.; Yang, J.; Charpentier, N.; LeCluyse, E. L.; Black, C.; Yang, D.; Shi, D.; Yan, B. Antiplatelet agents aspirin and clopidogrel are hydrolyzed by distinct carboxylesterases, and clopidogrel is transesterificated in the presence of ethyl alcohol. *J. Pharmacol. Exp. Ther.* **2006***, 319*, 1467-1476.

57. Bouman, H. J.; Schömig, E.; Van Werkum, J. W.; Velder, J.; Hackeng, C. M.; Hirschhäuser, C.; Waldmann, C.; Schmalz, H. -.; Ten Berg, J. M.; Taubert, D. Paraoxonase-1 is a major determinant of clopidogrel efficacy. *Nat. Med.* **2011***, 17*, 110-116.

58. Achour, B.; Barber, J.; Rostami-Hodjegan, A. Expression of hepatic drug-metabolizing cytochrome P450 enzymes and their intercorrelations: A meta-analysis. *Drug Metab. Dispos.* **2014***, 42*, 1349-1356.

59. Tseng, E.; Walsky, R. L.; Luzietti Jr., R. A.; Harris, J. J.; Kosa, R. E.; Goosen, T. C.; Zientek, M. A.; Obach, R. S. Relative contributions of cytochrome CYP3A4 versus CYP3A5 for CYP3A-cleared drugs assessed in vitro using a CYP3A4-selective inactivator (CYP3cide). *Drug Metab. Dispos.* **2014***, 42*, 1163-1173.

60. Kato, Y.; Izukawa, T.; Oda, S.; Fukami, T.; Finel, M.; Yokoi, T.; Nakajima, M. Human UDP-glucuronosyltransferase (ugt) 2b10 in drug n-glucuronidation: Substrate screening and comparison with UGT1A3 and UGT1A4. *Drug Metab. Dispos.* **2013***, 41*, 1389-1397.

61. Gill, K. L.; Houston, J. B.; Galetin, A. Characterization of in vitro glucuronidation clearance of a range of drugs in human kidney microsomes: Comparison with liver and intestinal glucuronidation and impact of albumin. *Drug Metab. Dispos.* **2012***, 40*, 825-835.

62. Hiraoka, H.; Yamamoto, K.; Miyoshi, S.; Morita, T.; Nakamura, K.; Kadoi, Y.; Kunimoto, F.; Horiuchi, R. Kidneys contribute to the extrahepatic clearance of propofol in humans, but not lungs and brain. *Br. J. Clin. Pharmacol.* **2005***, 60*, 176-182.

63. Aleksa, K.; Matsell, D.; Krausz, K.; Gelboin, H.; Ito, S.; Koren, G. Cytochrome P450 3A and 2B6 in the developing kidney: Implications for ifosfamide nephrotoxicity. *Pediatr. Nephrol.* **2005***, 20*, 872-885.

64. Knights, K. M.; Rowland, A.; Miners, J. O. Renal drug metabolism in humans: The potential for drug-endobiotic interactions involving cytochrome P450 (CYP) and UDP-glucuronosyltransferase (UGT). *Br. J. Clin. Pharmacol.* **2013***, 76*, 587-602.

65. Court, M. H.; Duan, S. X.; Hesse, L. M.; Venkatakrishnan, K.; Greenblatt, D. J. Cytochrome P-450 2B6 is responsible for interindividual variability of propofol hydroxylation by human liver microsomes. *Anesthesiology* **2001***, 94*, 110-119.

66. Hediger, M. A.; Clémençon, B.; Burrier, R. E.; Bruford, E. A. The ABCs of membrane transporters in health and disease (SLC series): Introduction. *Mol. Asp. Med.* **2013***, 34*, 95-107.

67. Vasiliou, V.; Vasiliou, K.; Nebert, D. W. Human ATP-binding cassette (ABC) transporter family. *Hum. Genomics* **2009***, 3*, 281-290.

68. Roth, M.; Obaidat, A.; Hagenbuch, B. OATPs, OATs and OCTs: The organic anion and cation transporters of the SLCO and SLC22A gene superfamilies. *Br. J. Pharmacol.* **2012***, 165*, 1260-1287.

69. Hillgren, K. M.; Keppler, D.; Zur, A. A.; Giacomini, K. M.; Stieger, B.; Cass, C. E.; Zhang, L. Emerging transporters of clinical importance: An update from the international transporter consortium. *Clin. Pharmacol. Ther.* **2013***, 94*, 52-63.

70. Robey, R. W.; Pluchino, K. M.; Hall, M. D.; Fojo, A. T.; Bates, S. E.; Gottesman, M. M. Revisiting the role of ABC transporters in multidrug-resistant cancer. *Nat. Rev. Cancer* **2018***, 18*, 452-464.

71. Joshi, A. A.; Vaidya, S. S.; St-Pierre, M. V.; Mikheev, A. M.; Desino, K. E.; Nyandege, A. N.; Audus, K. L.; Unadkat, J. D.; Gerk, P. M. Placental ABC Transporters: Biological Impact and Pharmaceutical Significance. *Pharm. Res.* **2016***, 33*, 2847-2878.

72. Kimoto, E.; Mathialagan, S.; Tylaska, L.; Niosi, M.; Lin, J.; Carlo, A. A.; Tess, D. A.; Varma, M. V. S. Organic anion transporter 2-mediated hepatic uptake contributes to the clearance of

high-permeability-low-molecular-weight acid and zwitterion drugs: Evaluation using 25 drugs. *J. Pharmacol. Exp. Ther.* **2018***, 367*, 322-334.

73. Zamek-Gliszczynski, M. J.; Taub, M. E.; Chothe, P. P.; Chu, X.; Giacomini, K. M.; Kim, R. B.; Ray, A. S.; Stocker, S. L.; Unadkat, J. D.; Wittwer, M. B.; et al. Transporters in Drug Development: 2018 ITC Recommendations for Transporters of Emerging Clinical Importance. *Clin. Pharmacol. Ther.* **2018***, 104*, 890-899.
74. Mathialagan, S.; Costales, C.; Tylaska, L.; Kimoto, E.; Vildhede, A.; Johnson, J.; Johnson, N.; Sarashina, T.; Hashizume, K.; Isringhausen, C. D.; et al. In vitro studies with two human organic anion transporters: OAT2 and OAT7. *Xenobiotica* **2018***, 48*, 1037-1049.
75. Köck, K.; Brouwer, K. L. R. A perspective on efflux transport proteins in the liver. *Clin. Pharmacol. Ther.* **2012***, 92*, 599-612.
76. Guo, Y.; Chu, X.; Parrott, N. J.; Brouwer, K. L. R.; Hsu, V.; Nagar, S.; Matsson, P.; Sharma, P.; Snoeys, J.; Sugiyama, Y.; et al. Advancing Predictions of Tissue and Intracellular Drug Concentrations Using In Vitro, Imaging and Physiologically Based Pharmacokinetic Modeling Approaches. *Clin. Pharmacol. Ther.* **2018***, 104*, 865-889.
77. Drozdzik, M.; Busch, D.; Lapczuk, J.; Müller, J.; Ostrowski, M.; Kurzawski, M.; Oswald, S. Protein Abundance of Clinically Relevant Drug Transporters in the Human Liver and Intestine: A Comparative Analysis in Paired Tissue Specimens. *Clin. Pharmacol. Ther.* **2019***, 105*, 1204-1212.
78. Drozdzik, M.; Gröer, C.; Penski, J.; Lapczuk, J.; Ostrowski, M.; Lai, Y.; Prasad, B.; Unadkat, J. D.; Siegmund, W.; Oswald, S. Protein abundance of clinically relevant multidrug transporters along the entire length of the human intestine. *Mol. Pharm.* **2014***, 11*, 3547-3555.
79. Ming, X.; Thakker, D. R. Role of basolateral efflux transporter MRP4 in the intestinal absorption of the antiviral drug adefovir dipivoxil. *Biochem. Pharmacol.* **2010***, 79*, 455-462.
80. De Waart, D. R.; Van De Wetering, K.; Kunne, C.; Duijst, S.; Paulusma, C. C.; Oude Elferink, R. P. J. Oral availability of cefadroxil depends on ABCC3 and ABCC4. *Drug Metab. Dispos.* **2012***, 40*, 515-521.
81. Johnson, M.; Patel, D.; Matheny, C.; Ho, M.; Chen, L.; Ellens, H. Inhibition of intestinal OATP2B1 by the calcium receptor antagonist ronacaleret results in a significant drug-drug interaction by causing a 2-fold decrease in exposure of rosuvastatin. *Drug Metab. Dispos.* **2017***, 45*, 27-34.
82. McFeely, S. J.; Wu, L.; Ritchie, T. K.; Unadkat, J. Organic anion transporting polypeptide 2B1 – More than a glass-full of drug interactions. *Pharmacol. Ther.* **2019***, 196*, 204-215.
83. Burt, H. J.; Riedmaier, A. E.; Harwood, M. D.; Crewe, H. K.; Gill, K. L.; Neuhoff, S. Abundance of hepatic transporters in Caucasians: A meta-analysis. *Drug Metab. Dispos.* **2016***, 44*, 1550-1561.
84. Aleo, M. D.; Shah, F.; He, K.; Bonin, P. D.; Rodrigues, A. D. Evaluating the Role of Multidrug Resistance Protein 3 (MDR3) Inhibition in Predicting Drug-Induced Liver Injury Using 125 Pharmaceuticals. *Chem. Res. Toxicol.* **2017***, 30*, 1219-1229.
85. Slot, A. J.; Molinski, S. V.; Cole, S. P. C. Mammalian multidrug-resistance proteins (MRPs). *Essays Biochem.* **2011***, 50*, 179-207.
86. Basit, A.; Radi, Z.; Vaidya, V. S.; Karasu, M.; Prasad, B. Kidney Cortical Transporter Expression across Species Using Quantitative Proteomics. *Drug Metab. Dispos.* **2019***, 47*, 802-808.
87. Cheung, K. W. K.; van Groen, B. D.; Spaans, E.; van Borselen, M. D.; de Bruijn, A. C. J. M.; Simons-Oosterhuis, Y.; Tibboel, D.; Samsom, J. N.; Verdijk, R. M.; Smeets, B.; et al. A Comprehensive Analysis of Ontogeny of Renal Drug Transporters: mRNA Analyses, Quantitative Proteomics, and Localization. *Clin. Pharmacol. Ther.* **2019**.
88. Prasad, B.; Johnson, K.; Billington, S.; Lee, C.; Chung, G. W.; Brown, C. D. A.; Kelly, E. J.; Himmelfarb, J.; Unadkat, J. D. Abundance of drug transporters in the human kidney cortex as quantified by quantitative targeted proteomics. *Drug Metab. Dispos.* **2016***, 44*, 1920-1924.
89. Uchida, Y.; Ohtsuki, S.; Katsukura, Y.; Ikeda, C.; Suzuki, T.; Kamiie, J.; Terasaki, T. Quantitative targeted absolute proteomics of human blood-brain barrier transporters and receptors. *J. Neurochem.* **2011***, 117*, 333-345.

90. Obach, R. S. Pharmacologically active drug metabolites: Impact on drug discovery and pharmacotherapy. *Pharmacol. Rev.* **2013**, *65*, 578-640.

91. Penner, N.; Xu, L.; Prakash, C. Radiolabeled absorption, distribution, metabolism, and excretion studies in drug development: Why, when, and how? *Chem. Res. Toxicol.* **2012**, *25*, 513-531.

92. Fagerholm, U. Prediction of human pharmacokinetics - Biliary and intestinal clearance and enterohepatic circulation. *J. Pharm. Pharmacol.* **2008**, *60*, 535-542.

93. Zhang, D.; Frost, C. E.; He, K.; Rodrigues, A. D.; Wang, X.; Wang, L.; Goosen, T. C.; Humphreys, W. G. Investigating the enteroenteric recirculation of apixaban, a factor Xa inhibitor: Administration of activated charcoal to bile duct-cannulated rats and dogs receiving an intravenous dose and use of drug transporter knockout rats. *Drug Metab. Dispos.* **2013**, *41*, 906-915.

94. Ichida, K.; Matsuo, H.; Takada, T.; Nakayama, A.; Murakami, K.; Shimizu, T.; Yamanashi, Y.; Kasuga, H.; Nakashima, H.; Nakamura, T.; Takada, Y.; Kawamura, Y.; Inoue, H.; Okada, C.; Utsumi, Y.; Ikebuchi, Y.; Ito, K.; Nakamura, M.; Shinohara, Y.; Hosoyamada, M.; Sakurai, Y.; Shinomiya, N.; Hosoya, T.; Suzuki, H. Decreased extra-renal urate excretion is a common cause of hyperuricemia. *Nat. Commun.* **2012**, *3*.

95. Rolan, P. E.; Mercer, A. J.; Tate, E.; Benjamin, I.; Posner, J. Disposition of atovaquone in humans. *Antimicrob. Agents Chemother.* **1997**, *41*, 1319-1321.

96. Lombardo, F.; Berellini, G.; Obach, R. S. Trend analysis of a database of intravenous pharmacokinetic parameters in humans for 1352 drug compounds. *Drug Metab. Dispos.* **2018**, *46*, 1466-1477.

97. Waring, M. J. Lipophilicity in drug discovery. *Expert Opin. Drug. Discov.* **2010**, *5*, 235-248.

98. Varma, M. V. S.; Feng, B.; Obach, R. S.; Troutman, M. D.; Chupka, J.; Miller, H. R.; El-Kattan, A. Physicochemical determinants of human renal clearance. *J. Med. Chem.* **2009**, *52*, 4844-4852.

99. Varma, M. V. S.; Chang, G.; Lai, Y.; Feng, B.; El-Kattan, A. F.; Litchfield, J.; Goosen, T. C. Physicochemical property space of hepatobiliary transport and computational models for predicting rat biliary excretion. *Drug Metab. Dispos.* **2012**, *40*, 1527-1537.

100. Hosey, C. M.; Broccatelli, F.; Benet, L. Z. Predicting when biliary excretion of parent drug is a major route of elimination in humans. *AAPS J.* **2014**, *16*, 1085-1096.

101. Yang, X.; Gandhi, Y. A.; Duignan, D. B.; Morris, M. E. Prediction of biliary excretion in rats and humans using molecular weight and quantitative structure-pharmacokinetic relationships. *AAPS Journal* **2009**, *11*, 511-525.

102. Luo, G.; Johnson, S.; Hsueh, M. -.; Zheng, J.; Cai, H.; Xin, B.; Chong, S.; He, K.; Harper, T. W. In silico prediction of biliary excretion of drugs in rats based on physicochemical properties. *Drug Metab. Dispos.* **2010**, *38*, 422-430.

103. Camenisch, G. P. Drug Disposition Classification Systems in Discovery and Development: A Comparative Review of the BDDCS, ECCS and ECCCS Concepts. *Pharm. Res.* **2016**, *33*, 2583-2593.

104. El-Kattan, A. F.; Varma, M. V. S. Navigating transporter sciences in pharmacokinetics characterization using the extended clearance classification system. *Drug Metab. Dispos.* **2018**, *46*, 729-739.

105. Mathialagan, S.; Piotrowski, M. A.; Tess, D. A.; Feng, B.; Litchfield, J.; Varma, M. V. Quantitative prediction of human renal clearance and drug-drug interactions of organic anion transporter substrates using in vitro transport data: A relative activity factor approach. *Drug Metab. Dispos.* **2017**, *45*, 409-417.

106. Schadt, S.; Bister, B.; Chowdhury, S. K.; Funk, C.; Hop, C. E. C. A.; Humphreys, W. G.; Igarashi, F.; Alexander, D. J.; Kagan, M.; Khojasteh, S. C.; et al.. A decade in the MIST: Learnings from investigations of drug metabolites in drug development under the "metabolites in safety testing" regulatory guidance. *Drug Metab. Dispos.* **2018**, *46*, 865-878.

107. Van Heek, M.; Farley, C.; Compton, D. S.; Hoos, L.; Alton, K. B.; Sybertz, E. J.; Davis Jr., H. R. Comparison of the activity and disposition of the novel cholesterol absorption inhibitor, SCH58235, and its glucuronide, SCH60663. *Br. J. Pharmacol.* **2000**, *129*, 1748-1754.

108. Sakamoto, S.; Kusuhara, H.; Miyata, K.; Shimaoka, H.; Kanazu, T.; Matsuo, Y.; Nomura, K.; Okamura, N.; Hara, S.; Horie, K.; Baba, T.; Sugiyama, Y. Glucuronidation converting methyl 1-(3,4-dimethoxyphenyl)-3-(3- ethylvaleryl)-4-hydroxy-6,7,8-trimethoxy-2-naphthoate (S-8921) to a potent apical sodium-dependent bile acid transporter inhibitor, resulting in a hypocholesterolemic action. *J. Pharmacol. Exp. Ther.* **2007***, 322*, 610-618.

109. Ma, Y.; Fu, Y.; Khojasteh, S. C.; Dalvie, D.; Zhang, D. Glucuronides as Potential Anionic Substrates of Human Cytochrome P450 2C8 (CYP2C8). *J. Med. Chem.* **2017***, 60*, 8691-8705.

110. Iwaki, M.; Shimada, H.; Irino, Y.; Take, M.; Egashira, S. Inhibition of methotrexate uptake via organic anion transporters OAT1 and OAT3 by glucuronides of nonsteroidal anti-inflammatory drugs. *Biol. Pharm. Bull.* **2017***, 40*, 926-931.

111. Loi, C. -.; Smith, D. A.; Dalvie, D. Which metabolites circulate? *Drug Metab. Dispos.* **2013***, 41*, 933-951.

112. Manners, C. N.; Payling, D. W.; Smith, D. A. Distribution coefficient, a convenient term for the relation of predictable physico-chemical properties to metabolic processes. *Xenobiotica* **1988***, 18*, 331-350.

113. Smith, D. A.; Dalvie, D. Why do metabolites circulate? *Xenobiotica* **2012***, 42*, 107-126.

114. Nguyen, H. Q.; Kimoto, E.; Callegari, E.; Obach, R. S. Mechanistic modeling to predict midazolam metabolite exposure from in vitro data. *Drug Metab. Dispos.* **2016***, 44*, 781-791.

115. Iegre, J.; Hayes, M. A.; Thompson, R. A.; Weidolf, L.; Isin, E. M. Database extraction of metabolite information of drug candidates: Analysis of 27 AstraZeneca compounds with human absorption, distribution, metabolism, and excretion data. *Drug Metab. Dispos.* **2016***, 44*, 732-740.

116. Bowers, G. D.; Culp, A.; Reese, M. J.; Tabolt, G.; Moss, L.; Piscitelli, S.; Huynh, P.; Wagner, D.; Ford, S. L.; Gould, E. P.; et al. Disposition and metabolism of cabotegravir: A comparison of biotransformation and excretion between different species and routes of administration in humans. *Xenobiotica* **2016***, 46*, 147-162.

117. Upthagrove, A.; Chen, J.; Meyers, C. D.; Kulmatycki, K.; Bretz, A.; Wang, L.; Peng, L.; Palamar, S.; Lin, M.; Majumdar, T.; Tran, P.; Einolf, H. J. Pradigastat disposition in humans: in vivo and in vitro investigations. *Xenobiotica* **2017***, 47*, 1077-1089.

118. Smith, D. A.; Brown, K.; Neale, M. G. Chromone-2-carboxylic acids: Roles of acidity and lipophilicity in drug disposition. *Drug Metab. Rev.* **1985***, 16*, 365-388.

119. Giorgi, E. P.; Stein, W. D. The transport of steroids into animal cells in culture. *Endocrinology* **1981***, 108*, 688-697.

120. Kimoto, E.; Li, R.; Scialis, R. J.; Lai, Y.; Varma, M. V. S. Hepatic Disposition of Gemfibrozil and Its Major Metabolite Gemfibrozil 1-O-ß-Glucuronide. *Mol. Pharm.* **2015***, 12*, 3943-3952.

121. Miyauchi, S.; Sugiyama, Y.; Iga, T.; Hanano, M. Membrane-limited hepatic transport of the conjugative metabolites of 4-methylumbelliferone in rats. *J. Pharm. Sci.* **1988***, 77*, 688-692.

122. Zhou, S.; Feng, X.; Kestell, P.; Paxton, J. W.; Baguley, B. C.; Chan, E. Transport of the investigational anti-cancer drug 5,6-dimethylxanthenone-4- acetic acid and its acyl glucuronide by human intestinal Caco-2 cells. *Eur. J. Pharm. Sci.* **2005***, 24*, 513-524.

123. Zhang, Y.; Han, Y. -.; Putluru, S. P.; Matta, M. K.; Kole, P.; Mandlekar, S.; Furlong, M. T.; Liu, T.; Iyer, R. A.; Marathe, P.; Yet al. Diclofenac and Its Acyl Glucuronide: Determination of in Vivo Exposure in Human Subjects and Characterization as Human Drug Transporter Substrates in Vitro. *Drug Metab. Dispos.* **2016***, 44*, 320-328.

124. Zhang, Q.; Zhang, Y.; Boer, J.; Shi, J. G.; Hu, P.; Diamond, S.; Yeleswaram, S. In vitro interactions of epacadostat and its major metabolites with human efflux and uptake transporters: Implications for pharmacokinetics and drug interactions. *Drug Metab. Dispos.* **2017***, 45*, 612-623.

125. Zamek-Gliszczynski, M. J.; Hoffmaster, K. A.; Nezasa, K. -.; Tallman, M. N.; Brouwer, K. L. R. Integration of hepatic drug transporters and phase II metabolizing enzymes: Mechanisms of hepatic excretion of sulfate, glucuronide, and glutathione metabolites. *Eur. J. Pharm. Sci.* **2006***, 27*, 447-486.

126. Nguyen, H. Q.; Lin, J.; Kimoto, E.; Callegari, E.; Tse, S.; Obach, R. S. Prediction of Losartan-Active Carboxylic Acid Metabolite Exposure Following Losartan Administration

Using Static and Physiologically Based Pharmacokinetic Models. *J. Pharm. Sci.* **2017***, 106*, 2758-2770.

127. Ulm, E.; Hichens, M.; Gomez, H.; Till, A.; Hand, E.; Vassil, T.; Biollaz, J.; Brunner, H.; Schelling, J. Enalapril maleate and a lysine analogue (MK-521): disposition in man. *Br. J. Clin. Pharmacol.* **1982***, 14*, 357-362.

128. de Lannoy, I. A. M.; Barker III, F.; Pang, K. S. Formed and preformed metabolite excretion clearances in liver, a metabolite formation organ: Studies on enalapril and enalaprilat in the single-pass and recirculating perfused rat liver. *J. Pharmacokinet. Biopharm.* **1993***, 21*, 395-422.

129. Zamek-Gliszczynski, M. J.; Chu, X.; Polli, J. W.; Paine, M. F.; Galetin, A. Understanding the transport properties of metabolites: Case studies and considerations for drug development. *Drug Metab. Dispos.* **2014***, 42*, 650-664.

130. Treyer, A.; Ullah, M.; Parrott, N.; Molitor, B.; Fowler, S.; Artursson, P. Impact of Intracellular Concentrations on Metabolic Drug-Drug Interaction Studies. *AAPS J.* **2019***, 21*.

131. Bauer, M.; Matsuda, A.; Wulkersdorfer, B.; Philippe, C.; Traxl, A.; Özvegy-Laczka, C.; Stanek, J.; Nics, L.; Klebermass, E. -.; Poschner, S.; et al. Influence of OATPs on Hepatic Disposition of Erlotinib Measured With Positron Emission Tomography. *Clin. Pharmacol. Ther.* **2018***, 104*, 139-147.

132. Shimizu, K.; Takashima, T.; Yamane, T.; Sasaki, M.; Kageyama, H.; Hashizume, Y.; Maeda, K.; Sugiyama, Y.; Watanabe, Y.; Senda, M. Whole-body distribution and radiation dosimetry of [11 C]telmisartan as a biomarker for hepatic organic anion transporting polypeptide (OATP) 1B3. *Nucl. Med. Biol.* **2012***, 39*, 847-853.

133. Vasilyeva, A.; Durmus, S.; Li, L.; Wagenaar, E.; Hu, S.; Gibson, A. A.; Panetta, J. C.; Mani, S.; Sparreboom, A.; Baker, S. D.; Schinkel, A. H. Hepatocellular shuttling and recirculation of sorafenib-glucuronide is dependent on Abcc2, Abcc3, and Oatp1a/1b. *Cancer Res.* **2015***, 75*, 2729-2736.

134. Smith, D. A.; Obach, R. S. Metabolites: Have we MIST out the importance of structure and physicochemistry? *Bioanalysis* **2010***, 2*, 1223-1232.

135. Sasaki, T.; Hirota, T.; Ryokai, Y.; Kobayashi, D.; Kimura, M.; Irie, S.; Higuchi, S.; Ieiri, I. Systematic screening of human ABCC3 polymorphisms and their effects on MRP3 expression and function. *Drug Metab. Pharmacokinet.* **2011***, 26*, 374-386.

136. Brouwer, K. L. R.; Keppler, D.; Hoffmaster, K. A.; Bow, D. A. J.; Cheng, Y.; Lai, Y.; Palm, J. E.; Stieger, B.; Evers, R. In vitro methods to support transporter evaluation in drug discovery and development. *Clin. Pharmacol. Ther.* **2013***, 94*, 95-112.

137. Slachtova, L.; Seda, O.; Behunova, J.; Mistrik, M.; Martasek, P. Genetic and biochemical study of dual hereditary jaundice: Dubin-Johnson and Gilbert's syndromes. Haplotyping and founder effect of deletion in ABCC2. *Eur. J. Hum. Genet.* **2016***, 24*, 704-709.

138. Kato, K.; Kusuhara, H.; Kumagai, Y.; Ieiri, I.; Mori, H.; Ito, S.; Nakai, Y.; Maeda, K.; Sugiyama, Y. Association of multidrug resistance-associated protein 2 single nucleotide polymorphism rs12762549 with the basal plasma levels of phase II metabolites of isoflavonoids in healthy Japanese individuals. *Pharmacogenet. Genomics* **2012***, 22*, 344-354.

139. Keppler, D.; Konig, J. Hepatic secretion of conjugated drugs and endogenous substances. *Semin. Liver Dis.* **2000***, 20*, 265-272.

140. Van Der Schoor, L. W. E.; Verkade, H. J.; Kuipers, F.; Jonker, J. W. New insights in the biology of ABC transporters ABCC2 and ABCC3: Impact on drug disposition. *Expert Opin. Drug Metab. Toxicol.* **2015***, 11*, 273-293.

141. Chu, X. -.; Liang, Y.; Cai, X.; Cuevas-Licea, K.; Rippley, R. K.; Kassahun, K.; Shou, M.; Braun, M. P.; Doss, G. A.; Anari, M. R.; Evers, R. Metabolism and renal elimination of gaboxadol in humans: Role of UDP-glucuronosyltransferases and transporters. *Pharm. Res.* **2009***, 26*, 459-468.

142. Gao, C.; Zhang, H.; Guo, Z.; You, T.; Chen, X.; Zhong, D. Mechanistic studies on the absorption and disposition of scutellarin in humans: Selective OATP2B1-mediated hepatic uptake is a likely key determinant for its unique pharmacokinetic characteristics. *Drug Metab. Dispos.* **2012***, 40*, 2009-2020.

143. Mizuno, N.; Takahashi, T.; Kusuhara, H.; Schuetz, J. D.; Niwa, T.; Sugiyama, Y. Evaluation of the role of Breast Cancer Resistance Protein (BCRP/ABCG2) and Multidrug Resistance-Associated Protein 4 (MRP4/ABCC4) in the urinary excretion of sulfate and glucuronide metabolites of edaravone (MCI-186; 3-methyl-1-phenyl-2-pyrazolin-5-one). *Drug Metab. Dispos.* **2007***, 35*, 2045-2052.

144. Mutsaers, H. A. M.; Caetano-Pinto, P.; Seegers, A. E. M.; Dankers, A. C. A.; van den Broek, P. H. H.; Wetzels, J. F. M.; van den Brand, J. A. J. G.; van den Heuvel, L. P.; Hoenderop, J. G.; Wilmer, M. J. G.; Masereeuw, R. Proximal tubular efflux transporters involved in renal excretion of p-cresyl sulfate and p-cresyl glucuronide: Implications for chronic kidney disease pathophysiology. *Toxicol. Vitro* **2015***, 29*, 1868-1877.

145. Wittgen, H. G. M.; Van Den Heuvel, J. J. M. W.; Van Den Broek, P. H. H.; Siissalo, S.; Groothuis, G. M. M.; De Graaf, I. A. M.; Koenderink, J. B.; Russel, F. G. M. Transport of the coumarin metabolite 7-hydroxycoumarin glucuronide is mediated via multidrug resistance-associated proteins 3 and 4. *Drug Metab. Dispos.* **2012***, 40*, 1076-1079.

146. Li, D.; Chen, L.; Li, Y.; Tian, S.; Sun, H.; Hou, T. ADMET evaluation in drug discovery. 13. Development of in silico prediction models for p-glycoprotein substrates. *Mol. Pharm.* **2014***, 11*, 716-726.

147. Lagas, J. S.; Sparidans, R. W.; Wagenaar, E.; Beijnen, J. H.; Schinkel, A. H. Hepatic clearance of reactive glucuronide metabolites of diclofenac in the mouse is dependent on multiple ATP-binding cassette efflux transporters. *Mol. Pharmacol.* **2010***, 77*, 687-694.

148. Zamek-Gliszczynski, M. J.; Nezasa, K. -.; Tian, X.; Kalvass, J. C.; Patel, N. J.; Raub, T. J.; Brouwer, K. L. R. The important role of Bcrp (Abcg2) in the biliary excretion of sulfate and glucuronide metabolites of acetaminophen, 4-methylumbelliferone, and harmol in mice. *Mol. Pharmacol.* **2006***, 70*, 2127-2133.

149. Zamek-Gliszczynski, M. J.; Nezasa, K. -.; Tian, X.; Bridges, A. S.; Lee, K.; Belinsky, M. G.; Kruh, G. D.; Brouwer, K. L. R. Evaluation of the role of multidrug resistance-associated protein (Mrp) 3 and Mrp4 in hepatic basolateral excretion of sulfate and glucuronide metabolites of acetaminophen, 4-methylumbelliferone, and harmol in Abcc3-/- and Abcc4-/- mice. *J. Pharmacol. Exp. Ther.* **2006***, 319*, 1485-1491.

150. Nishimura, M.; Naito, S. Tissue-specific mRNA expression profiles of human ATP-binding cassette and solute carrier transporter superfamilies. *Drug Metab. Pharmacokinet.* **2005***, 20*, 452-477.

151. Belinsky, M. G.; Chen, Z. -.; Shchaveleva, I.; Zeng, H.; Kruh, G. D. Characterization of the drug resistance and transport properties of multidrug resistance protein 6 (MRP6, ABCC6). *Cancer Res.* **2002***, 62*, 6172-6177.

152. Iliás, A.; Urbán, Z.; Seidl, T. L.; Saux, O. L.; Sinkó, E.; Boyd, C. D.; Sarkadi, B.; Váradi, A. Loss of ATP-dependent transport activity in pseudoxanthoma elasticum-associated mutants of human ABCC6 (MRP6). *J. Biol. Chem.* **2002***, 277*, 16860-16867.

153. Borst, P.; De Wolf, C.; Van De Wetering, K. Multidrug resistance-associated proteins 3, 4, and 5. *Pflug. Arch. Eur. J. Physiol.* **2007***, 453*, 661-673.

154. Cole, S. P. C. Multidrug resistance protein 1 (mrp1, abcc1), a "multitasking" atp-binding cassette (abc,) transporter. *J. Biol. Chem.* **2014***, 289*, 30880-30888.

155. Zheng, L.; Zhu, L.; Zhao, M.; Shi, J.; Li, Y.; Yu, J.; Jiang, H.; Wu, J.; Tong, Y.; Liu, Y.; Hu, M.; Lu, L.; Liu, Z. In Vivo Exposure of Kaempferol Is Driven by Phase II Metabolic Enzymes and Efflux Transporters. *AAPS J.* **2016***, 18*, 1289-1299.

156. Szafraniec, M. J.; Szczygiel, M.; Urbanska, K.; Fiedor, L. Determinants of the activity and substrate recognition of breast cancer resistance protein (ABCG2). *Drug Metab. Rev.* **2014***, 46*, 459-474.

157. Zamek-Gliszczynski, M. J.; Lee, C. A.; Poirier, A.; Bentz, J.; Chu, X.; Ellens, H.; Ishikawa, T.; Jamei, M.; Kalvass, J. C.; Nagar, S.; et al. ITC recommendations for transporter kinetic parameter estimation and translational modeling of transport-mediated PK and DDIs in humans. *Clin. Pharmacol. Ther.* **2013***, 94*, 64-79.

158. Hirouchi, M.; Kusuhara, H.; Onuki, R.; Ogilvie, B. W.; Parkinson, A.; Sugiyama, Y. Construction of triple-transfected cells [organic anion-transporting polypeptide (OATP) 1B1/multidrug resistance-associated protein (MRP) 2/MRP3 and OATP1B1/MRP2/MRP4]

for analysis of the sinusoidal function of MRP3 and MRP4. *Drug Metab. Dispos.* **2009***, 37*, 2103-2111.

159. Zamek-Gliszczynski, M. J.; Hoffmaster, K. A.; Humphreys, J. E.; Tian, X.; Nezasa, K. -.; Brouwer, K. L. R. Differential involvement of Mrp2 (Abcc2) and Bcrp (Abcg2) in biliary excretion of 4-methylumbelliferyl glucuronide and sulfate in the rat. *J. Pharmacol. Exp. Ther.* **2006***, 319*, 459-467.

160. Li, Y.; Song, W.; Ou, X.; Luo, G.; Xie, Y.; Sun, R.; Wang, Y.; Qi, X.; Hu, M.; Liu, Z.; Zhu, L. Breast cancer resistance protein and multidrug resistance protein 2 determine the disposition of esculetin-7-o-glucuronide and 4-methylesculetin-7-o-glucuronide. *Drug Metab. Dispos.* **2019**, 203-214.

161. Jiang, H.; Yu, J.; Zheng, H.; Chen, J.; Wu, J.; Qi, X.; Wang, Y.; Wang, X.; Hu, M.; Zhu, L.; Liu, Z. Breast Cancer Resistance Protein and Multidrug Resistance Protein 2 Regulate the Disposition of Acacetin Glucuronides. *Pharm. Res.* **2017***, 34*, 1402-1415.

162. Manautou, J. E.; De Waart, D. R.; Kunne, C.; Zelcer, N.; Goedken, M.; Borst, P.; Elferink, R. O. Altered disposition of acetaminophen in mice with a disruption of the Mrp3 gene. *Hepatology* **2005***, 42*, 1091-1098.

163. Xiong, H.; Turner, K. C.; Ward, E. S.; Jansen, P. L. M.; Brouwer, K. L. R. Altered hepatobiliary disposition of acetaminophen glucuronide in isolated perfused livers from multidrug resistance-associated protein 2-deficient TR - rats 1. *J. Pharmacol. Exp. Ther.* **2000***, 295*, 512-518.

164. Van De Steeg, E.; Stránecký, V.; Hartmannová, H.; Nosková, L.; Hřebíček, M.; Wagenaar, E.; Van Esch, A.; De Waart, D. R.; Oude Elferink, R. P. J.; Kenworthy, K. E.; et al. Complete OATP1B1 and OATP1B3 deficiency causes human Rotor syndrome by interrupting conjugated bilirubin reuptake into the liver. *J. Clin. Invest.* **2012***, 122*, 519-528.

165. Ji, J. Z.; Tai, T.; Huang, B. B.; Gu, T. T.; Mi, Q. Y.; Xie, H. G. Mrp3 Transports Clopidogrel Acyl Glucuronide from the Hepatocytes into Blood. *Drug Metab. Dispos.* **2018***, 46*, 151-154.

166. Scialis, R. J.; Aleksunes, L. M.; Csanaky, I. L.; Klaassen, C. D.; Manautou, J. E. Identification and Characterization of Efflux Transporters that Modulate the Subtoxic Disposition of Diclofenac and its Metabolites. *Drug Metab. Dispos.* **2019**.

167. Takenaka, O.; Horie, T.; Suzuki, H.; Sugiyama, Y. Different biliary excretion systems for glucuronide and sulfate of a model compound; Study using Eisai hyperbilirubinemic rats. *J. Pharmacol. Exp. Ther.* **1995***, 274*, 1362-1369.

168. Zamek-Gliszczynski, M. J.; Day, J. S.; Hillgren, K. M.; Phillips, D. L. Efflux transport is an important determinant of ethinylestradiol glucuronide and ethinylestradiol sulfate pharmacokinetics. *Drug Metab. Dispos.* **2011***, 39*, 1794-1800.

169. Lagas, J. S.; Fan, L.; Wagenaar, E.; Vlaming, M. L. H.; Van Tellingen, O.; Beijnen, J. H.; Schinkel, A. H. P-glycoprotein (P-gp/Abcb1), Abcc2, and Abcc3 determine the pharmacokinetics of etoposide. *Clin. Cancer Res.* **2010***, 16*, 130-140.

170. De Waart, D. R.; Vlaming, M. L. H.; Kunne, C.; Schinkel, A. H.; Oude Elferink, R. P. J. Complex pharmacokinetic behavior of ezetimibe depends on Abcc2, Abcc3, and Abcg2. *Drug Metab. Dispos.* **2009***, 37*, 1698-1702.

171. Álvarez, A. I.; Vallejo, F.; Barrera, B.; Merino, G.; Prieto, J. G.; Tomás-Barberán, F.; Espín, J. C. Bioavailability of the glucuronide and sulfate conjugates of genistein and daidzein in breast cancer resistance protein 1 knockout mice. *Drug Metab. Dispos.* **2011***, 39*, 2008-2012.

172. Yang, Z.; Zhu, W.; Gao, S.; Yin, T.; Jiang, W.; Hu, M. Breast cancer resistance protein (ABCG2) determines distribution of genistein phase II metabolites: Reevaluation of the roles of ABCG2 in the disposition of genistein. *Drug Metab. Dispos.* **2012***, 40*, 1883-1893.

173. Zelcer, N.; Van De Wetering, K.; Hillebrand, M.; Sarton, E.; Kuil, A.; Wielinga, P. R.; Tephly, T.; Dahan, A.; Beijnen, J. H.; Borst, P. Mice lacking multidrug resistance protein 3 show altered morphine pharmacokinetics and morphine-6-glucuronide antinociception. *Proc. Natl. Acad. Sci. U. S. A.* **2005***, 102*, 7274-7279.

174. Van De Wetering, K.; Zelcer, N.; Kuil, A.; Feddema, W.; Hillebrand, M.; Vlaming, M. L. H.; Schinkel, A. H.; Beijnen, J. H.; Borst, P. Multidrug resistance proteins 2 and 3 provide alternative routes for hepatic excretion of morphine-glucuronides. *Mol. Pharmacol.* **2007***, 72*, 387-394.

175. Vlaming, M. L. H.; Teunissen, S. F.; Van De Steeg, E.; Van Esch, A.; Wagenaar, E.; Brunsveld, L.; De Greef, T. F. A.; Rosing, H.; Schellens, J. H. M.; Beijnen, J. H.; Schinkel, A. H. Bcrp1;Mdr1a/b;Mrp2 Combination Knockout Mice: Altered Disposition of the Dietary Carcinogen PhIP (2-Amino-1-Methyl-6-Phenylimidazo[4,5-b]Pyridine) and Its Genotoxic Metabolites s. *Mol. Pharmacol.* **2014**, *85*, 520-530.

176. Alfaras, I.; Pérez, M.; Juan, M. E.; Merino, G.; Prieto, J. G.; Planas, J. M.; Álvarez, A. I. Involvement of breast cancer resistance protein (BCRP1/ABCG2) in the bioavailability and tissue distribution of trans- resveratrol in knockout mice. *J. Agric. Food Chem.* **2010**, *58*, 4523-4528.

177. Van De Wetering, K.; Burkon, A.; Feddema, W.; Bot, A.; De Jonge, H.; Somoza, V.; Borst, P. Intestinal breast cancer resistance protein (BCRP)/Bcrp1 and multidrug resistance protein 3 (MRP3)/Mrp3 are involved in the pharmacokinetics of resveratrol. *Mol. Pharmacol.* **2009**, *75*, 876-885.

178. Sakamoto, S.; Kusuhara, H.; Horie, K.; Takahashi, K.; Baba, T.; Ishizaki, J.; Sugiyama, Y. Identification of the transporters involved in the hepatobiliary transport and intestinal efflux of methyl 1-(3,4-dimethoxyphenyl)-3-(3-ethylvaleryl)-4- hydroxy-6,7,8-trimethoxy-2-naphthoate (S-8921) glucuronide, a pharmacologically active metabolite of S-8921. *Drug Metab. Dispos.* **2008**, *36*, 1553-1561.

179. Chu, X.; Bleasby, K.; Evers, R. Species differences in drug transporters and implications for translating preclinical findings to humans. *Expert Opin. Drug Metab. Toxicol.* **2013**, *9*, 237-252.

180. Zamek-Gliszczynski, M. J.; Kalvass, J. C.; Pollack, G. M.; Brouwer, K. L. R. Relationship between drug/metabolite exposure and impairment of excretory transport function. *Drug Metab. Dispos.* **2009**, *37*, 386-390.

181. Shackleford, D. M.; Nation, R. L.; Milne, R. W.; Hayball, P. J.; Evans, A. M. Stereoselective hepatic disposition of model diastereomeric acyl glucuronides. *J. Pharmacokinet. Pharmacodyn.* **2004**, *31*, 1-27.

182. Takenaka, O.; Horie, T.; Suzuki, H.; Sugiyama, Y. Carrier-mediated active transport of the glucuronide and sulfate of 6- hydroxy-5,7-dimethyl-2-methylamino-4-(3-pyridylmethyl) benzothiazole (E3040) into rat liver: Quantitative comparison of permeability in isolated hepatocytes, perfused liver and liver in vivo. *J. Pharmacol. Exp. Ther.* **1997**, *280*, 948-958.

183. Patel, M.; Christian Eberl, H.; Wolf, A.; Pierre, E.; Polli, J. W.; Zamek-Gliszczynski, M. J. Mechanistic basis of cabotegravir–glucuronide disposition in humanss. *J. Pharmacol. Exp. Ther.* **2019**, *370*, 269-277.

184. Zhong, K.; Li, X.; Xie, C.; Zhang, Y.; Zhong, D.; Chen, X. Effects of renal impairment on the pharmacokinetics of morinidazole: Uptake transporter-mediated renal clearance of the conjugated metabolites. *Antimicrob. Agents Chemother.* **2014**, *58*, 4153-4161.

185. Fu, Q.; Chen, M.; Anderson, J. T.; Sun, X.; Hu, S.; Sparreboom, A.; Baker, S. D. Interaction Between Sex and Organic Anion-Transporting Polypeptide 1b2 on the Pharmacokinetics of Regorafenib and Its Metabolites Regorafenib-N-Oxide and Regorafenib-Glucuronide in Mice. *Clin. Transl. Sci.* **2019**, *12*, 400-407.

186. Zeng, M.; Sun, R.; Basu, S.; Ma, Y.; Ge, S.; Yin, T.; Gao, S.; Zhang, J.; Hu, M. Disposition of flavonoids via recycling: Direct biliary excretion of enterically or extrahepatically derived flavonoid glucuronides. *Mol. Nutr. Food Res.* **2016**, *60*, 1006-1019.

187. Luukkanen, L.; Mikkola, J.; Forsman, T.; Taavitsainen, P.; Taskinen, J.; Elovaara, E. Glucuronidation of 1-hydroxypyrene by human liver microsomes and human UDP-glucuronosyltransferases UGT1A6, UGT1A7, and UGT1A9: Development of a high-sensitivity glucuronidation assay for human tissue. *Drug Metab. Dispos.* **2001**, *29*, 1096-1101.

188. Kidron, H.; Wissel, G.; Manevski, N.; Häkli, M.; Ketola, R. A.; Finel, M.; Yliperttula, M.; Xhaard, H.; Urtti, A. Impact of probe compound in MRP2 vesicular transport assays. *Eur. J. Pharm. Sci.* **2012**, *46*, 100-105.

189. Sjöstedt, N.; Holvikari, K.; Tammela, P.; Kidron, H. Inhibition of Breast Cancer Resistance Protein and Multidrug Resistance Associated Protein 2 by Natural Compounds and Their Derivatives. *Mol. Pharm.* **2017**, *14*, 135-146.

190. Sjöstedt, N.; Deng, F.; Rauvala, O.; Tepponen, T.; Kidron, H. Interaction of Food Additives with Intestinal Efflux Transporters. *Mol. Pharm.* **2017***, 14*, 3824-3833.

191. Chu, X. -.; Huskey, S. -. W.; Braun, M. P.; Sarkadi, B.; Evans, D. C.; Evers, R. Transport of Ethinylestradiol Glucuronide and Ethinylestradiol Sulfate by the Multidrug Resistance Proteins MRP1, MRP2, and MRP3. *J. Pharmacol. Exp. Ther.* **2004***, 309*, 156-164.

192. Herédi-Szabó, K.; Palm, J. E.; Andersson, T. B.; Pál, Á; Méhn, D.; Fekete, Z.; Beéry, E.; Jakab, K. T.; Jani, M.; Krajcsi, P. A P-gp vesicular transport inhibition assay - Optimization and validation for drug-drug interaction testing. *Eur. J. Pharm. Sci.* **2013***, 49*, 773-781.

193. Telbisz, Á; Müller, M.; Özvegy-Laczka, C.; Homolya, L.; Szente, L.; Váradi, A.; Sarkadi, B. Membrane cholesterol selectively modulates the activity of the human ABCG2 multidrug transporter. *Biochim. Biophys. Acta Biomembr.* **2007***, 1768*, 2698-2713.

194. Tepponen, T. Master's thesis: MDR1-transportterin inhibition tutkiminen Sf9-MDR1-vesikkeleissä. **2017** (available at *http://helda.helsinki.fi/handle/10138/230252*).

195. Chen, Z. -.; Lee, K.; Kruh, G. D. Transport of cyclic nucleotides and estradiol 17-ß-D-glucuronide by multidrug resistance protein 4. Resistance to 6-mercaptopurine and 6-thioguanine. *J. Biol. Chem.* **2001***, 276*, 33747-33754.

196. Elsby, R.; Smith, V.; Fox, L.; Stresser, D.; Butters, C.; Sharma, P.; Surry, D. D. Validation of membrane vesicle-based breast cancer resistance protein and multidrug resistance protein 2 assays to assess drug transport and the potential for drug-drug interaction to support regulatory submissions. *Xenobiotica* **2011***, 41*, 764-783.

197. Zeng, H.; Liu, G.; Rea, P. A.; Kruh, G. D. Transport of amphipathic anions by human multidrug resistance protein 3. *Cancer Res.* **2000***, 60*, 4779-4784.

198. Zelcer, N.; Saeki, T.; Reid, G.; Beijnen, J. H.; Borst, P. Characterization of Drug Transport by the Human Multidrug Resistance Protein 3 (ABCC3). *J. Biol. Chem.* **2001***, 276*, 46400-46407.

199. Zelcer, N.; Wetering, K. V. D.; Waart, R. D.; Scheffer, G. L.; Marschall, H. -.; Wielinga, P. R.; Kuil, A.; Kunne, C.; Smith, A.; Valk, M. V. D.; Wijnholds, J.; Elferink, R. O.; Borst, P. Mice lacking Mrp3 (Abcc3) have normal bile salt transport, but altered hepatic transport of endogenous glucuronides. *J. Hepatol.* **2006***, 44*, 768-775.

200. van de Wetering, K.; Feddema, W.; Helms, J. B.; Brouwers, J. F.; Borst, P. Targeted Metabolomics Identifies Glucuronides of Dietary Phytoestrogens as a Major Class of MRP3 Substrates In Vivo. *Gastroenterology* **2009***, 137*, 1725-1735.

201. Vaidya, S. S.; Walsh, S. W.; Gerk, P. M. Formation and efflux of ATP-binding cassette transporter substrate 2,4-dinitrophenyl-S-glutathione from cultured human term placental villous tissue fragments. *Mol. Pharm.* **2009***, 6*, 1689-1702.

202. Kato, Y.; Takahara, S.; Kato, S.; Kubo, Y.; Sai, Y.; Tamai, I.; Yabuuchi, H.; Tsuji, A. Involvement of multidrug resistance-associated protein 2 (Abcc2) in molecular weight-dependent biliary excretion of ß-lactam antibiotics. *Drug Metab. Dispos.* **2008***, 36*, 1088-1096.

203. Reid, G.; Wielinga, P.; Zelcer, N.; Van der Heijden, I.; Kuil, A.; De Haas, M.; Wijnholds, J.; Borst, P. The human multidrug resistance protein MRP4 functions as a prostaglandin efflux transporter and is inhibited by nonsteroidal anti inflammatory drugs. *Proc. Natl. Acad. Sci. U. S. A.* **2003***, 100*, 9244-9249.

204. Zelcer, N.; Reid, G.; Wielinga, P.; Kuil, A.; Van Der Heijden, I.; Schuetz, J. D.; Borst, P. Steroid and bile acid conjugates are substrates of human multidrug-resistance protein (MRP) 4 (ATP-binding cassette C4). *Biochem. J.* **2003***, 371*, 361-367.

205. Kalapos-Kovács, B.; Magda, B.; Jani, M.; Fekete, Z.; Szabõ, P. T.; Antal, I.; Krajcsi, P.; Klebovich, I. Multiple ABC Transporters Efflux Baicalin. *Phytother. Res.* **2015***, 29*, 1987-1990.

206. Wei, Y.; Wu, B.; Jiang, W.; Yin, T.; Jia, X.; Basu, S.; Yang, G.; Hu, M. Revolving door action of breast cancer resistance protein (BCRP) facilitates or controls the efflux of flavone glucuronides from UGT1A9-overexpressing hela cells. *Mol. Pharm.* **2013***, 10*, 1736-1750.

207. Yoshikawa, M.; Ikegami, Y.; Sano, K.; Yoshida, H.; Mitomo, H.; Sawada, S.; Ishikawa, T. Transport of SN-38 by the Wild Type of Human ABC Transporter ABCG2 and Its Inhibition by Quercetin, a Natural Flavonoid. *J. Exp. Ther. Oncol.* **2004***, 4*, 25-35.

208. Taylor, N. M. I.; Manolaridis, I.; Jackson, S. M.; Kowal, J.; Stahlberg, H.; Locher, K. P. Structure of the human multidrug transporter ABCG2. *Nature* **2017***, 546*, 504-509.

209. Mao, Q.; Unadkat, J. D. Role of the Breast Cancer Resistance Protein (BCRP/ABCG2) in Drug Transport—an Update. *AAPS J.* **2015***, 17*, 65-82.

210. Suzuki, M.; Suzuki, H.; Sugimoto, Y.; Sugiyama, Y. ABCG2 transports sulfated conjugates of steroids and xenobiotics. *J. Biol. Chem.* **2003***, 278*, 22644-22649.

211. Van De Wetering, K.; Sapthu, S. ABCG2 functions as a general phytoestrogen sulfate transporter in vivo. *FASEB J.* **2012***, 26*, 4014-4024.

212. Huang, L.; Hoffman, T.; Vore, M. Adenosine triphosphate-dependent transport of estradiol-17ß (ß-D- glucuronide) in membrane vesicles by MDR1 expressed in insect cells. *Hepatology* **1998***, 28*, 1371-1377.

213. Sjöstedt, N.; Salminen, T. A.; Kidron, H. Endogenous, cholesterol-activated ATP-dependent transport in membrane vesicles from Spodoptera frugiperda cells. *Eur. J. Pharm. Sci.* **2019***, 137*.

214. Elsby, R.; Martin, P.; Surry, D.; Sharma, P.; Fenner, K. Solitary Inhibition of the Breast Cancer Resistance Protein Efflux Transporter Results in a Clinically Significant Drug-Drug Interaction with Rosuvastatin by Causing up to a 2-Fold Increase in Statin Exposure. *Drug Metab. Dispos.* **2016***, 44*, 398-408.

215. Wang, L.; Prasad, B.; Salphati, L.; Chu, X.; Gupta, A.; Hop, C. E. C. A.; Evers, R.; Unadkat, J. D. Interspecies variability in expression of hepatobiliary transporters across human, dog, monkey, and rat as determined by quantitative proteomics. *Drug Metab. Dispos.* **2015***, 43*, 367-374.

216. Mizuno, N.; Suzuki, M.; Kusuhara, H.; Suzuki, H.; Takeuchi, K.; Niwa, T.; Jonker, J. W.; Sugiyama, Y. Impaired renal excretion of 6-hydroxy-5,7-dimethyl-2-methylamino-4-(3-pyridylmethyl) benzothiazole (E3040) sulfate in breast cancer resistance protein (BCRP1/ABCG2) knockout mice. *Drug Metab. Dispos.* **2004***, 32*, 898-901.

217. Sandberg, A. A.; Slaunwhite Jr., W. R. Studies on phenolic steroids in human subjects. II. The metabolic fate and hepato-biliary-enteric circulation of C14-estrone and C14-estradiol in women. *J. Clin. Invest.* **1957***, 36*, 1266-1278.

218. Sandberg, A. A.; Slaunwhite Jr., W. R. Studies on phenolic steroids in human subjects. VII. Metabolic fate of Estriol and Its Glucuronide. *J. Clin. Invest.* **1965***, 44*, 694-702.

219. Sandberg, A. A.; Slaunwhite Jr., W. R. Metabolism of 4-C14-testosterone in human subjects. I. Distribution in bile, blood, feces and urine. *J. Clin. Invest.* **1956***, 35*, 1331-1339.

220. Slaunwhite Jr., W. R.; Sandberg, A. A. Metabolism of 4-C14-testosterone in human subjects. III. Fate of androsterone and etiocholanolone. *J. Clin. Endocrinol. Metab.* **1958***, 18*, 1056-1066.

221. Iusuf, D.; Van De Steeg, E.; Schinkel, A. H. Hepatocyte hopping of oatp1b substrates contributes to efficient hepatic detoxification. *Clin. Pharmacol. Ther.* **2012***, 92*, 559-562.

222. Inoue, N.; Sandberg, A. A.; Graham, J. B.; Slaunwhite Jr., W. R. Studies on phenolic steroids in human subjects. 8. Metabolism of estriol-16 alpha-glucosiduronate. *J. Clin. Invest.* **1969***, 48*, 380-389.

223. Musey, P. I.; Green, R. N.; Hobkirk, R. The role of an enterohepatic system in the metabolism of 17ß-estradiol-17-glucosiduronate in the human female. *J. Clin. Endocrinol. Metab.* **1972***, 35*, 448-457.

224. Kirdani, R. Y.; Slaunwhite Jr., W. R.; Sandberg, A. A. Studies on phenolic steroids in human subjects. XIV. Studies on the fate of injected 6,7-H3-oestriol-3-C14-glucosiduronate. *J. Steroid Biochem.* **1970***, 1*, 265-278.

225. Siiteri, P. K.; Lieberman, S. In Vivo Studies with Radioactive Steroid Conjugates. I. The Fate of Randomly Tritiated Androsterone Glucuronoside in Humans. *Biochemistry* **1963***, 2*, 1171-1177.

226. Hobkirk, R.; Nilsen, M. Metabolism of estrone-3-glucosiduronate and 17ß-estradiol-3-glucosiduronate in the human female. *Steroids* **1970***, 15*, 649-667.

227. Robel, P.; Emiliozzi, R.; Baulieu, E. E. Studies on testosterone metabolism. V. Testosterone-3-H 17-glucuronide-14-C to 5-beta-androstane-3-alpha, 17-beta-diol-3-H 17-glucuronide-14-

C, the "direct" 5-beta-metabolism of testosterone glucuronide. *J. Biol. Chem.* **1966**, *241*, 5879-5885.

228. Garrett, E. R.; Venitz, J.; Eberst, K.; Cerda, J. J. Pharmacokinetics and bioavailabilities of hymecromone in human volunteers. *Biopharm. Drug Dispos.* **1993**, *14*, 13-39.

229. Armitage, A. K.; Houseman, T. H.; Lewis, P. J. Absorption and Metabolism of Nicotine from Cigarettes. *Brit. Med. J.* **1975**, *4*, 313-316.

230. Benowitz, N. L.; Jacob III, P. Trans-3'-hydroxycotinine: Disposition kinetics, effects and plasma levels during cigarette smoking. *Br. J. Clin. Pharmacol.* **2001**, *51*, 53-59.

231. Paterson, J. W.; Conolly, M. E.; Dollery, C. T.; Hayes, A.; Cooper, R. G. The pharmacodynamics and metabolism of propranolol in man. *Pharmacol. Clin.* **1970**, *2*, 127-133.

232. Walle, T.; Walle, U. K.; Olanoff, L. S. Quantitative account of propranolol metabolism in urine of normal man. *Drug Metab. Dispos.* **1985**, *13*, 204-209.

233. Stone, W. J.; Walle, T. Massive propranolol metabolite retention during maintenance hemodialysis. *Clin. Pharmacol. Ther.* **1980**, *28*, 449-455.

234. Alexander, S.; Stavric, V.; Smuk, M.; Sugar, J.; Schwers, J. Renal clearance of estriol and its conjugates in normal and abnormal pregnancies. *J. Clin. Endocrinol. Metab.* **1979**, *49*, 588-593.

235. Kellie, A. E.; Smith, E. R. Renal clearance of 17-oxo steroid conjugates found in human peripheral plasma. *Biochem. J.* **1957**, *66*, 490-495.

236. Walle, T.; Walle, U. K.; Cowart, T. D.; Conradi, E. C.; Gaffney, T. E. Selective induction of propranolol metabolism by smoking: additional effects on renal clearance of metabolites. *J. Pharmacol. Exp. Ther.* **1987**, *241*, 928-933.

237. Sneitz, N.; Vahermo, M.; Mosorin, J.; Laakkonen, L.; Poirier, D.; Finel, M. Regiospecificity and stereospecificity of human udpglucuronosyltransferases in the glucuronidation of estriol, 16-epiestriol, 17-epiestriol, and 13-epiestradiols. *Drug Metab. Dispos.* **2013**, *41*, 582-591.

238. Uchaipichat, V.; Mackenzie, P. I.; Guo, X. -.; Gardner-Stephen, D.; Galetin, A.; Houston, J. B.; Miners, J. O. Human UDP-glucuronosyltransferases: Isoform selectivity and kinetics of 4-methylumbelliferone and 1-naphthol glucuronidation, effects of organic solvents, and inhibition by diclofenac and probenecid. *Drug Metab. Dispos.* **2004**, *32*, 413-423.

239. Dickens, D.; Rädisch, S.; Chiduza, G. N.; Giannoudis, A.; Cross, M. J.; Malik, H.; Schaeffeler, E.; Sison-Young, R. L.; Wilkinson, E. L.; Goldring, C. E.; et al.. Cellular Uptake of the Atypical Antipsychotic Clozapine Is a Carrier-Mediated Process. *Mol. Pharm.* **2018**, *15*, 3557-3572.

240. Patel, M.; Johnson, M.; Sychterz, C. J.; Lewis, G. J.; Watson, C.; Ellens, H.; Polli, J. W.; Zamek-Gliszczynski, M. J. Hepatobiliary disposition of atovaquone: A case of mechanistically unusual biliary clearance. *J. Pharmacol. Exp. Ther.* **2018**, *366*, 37-45.

241. Shin, H. J.; Anzai, N.; Enomoto, A.; He, X.; Kim, D. K.; Endou, H.; Kanai, Y. Novel liver-specific organic anion transporter OAT7 that operates the exchange of sulfate conjugates for short chain fatty acid butyrate. *Hepatology* **2007**, *45*, 1046-1055.

242. Wong, C. C.; Barron, D.; Orfila, C.; Dionisi, F.; Krajcsi, P.; Williamson, G. Interaction of hydroxycinnamic acids and their conjugates with organic anion transporters and ATP-binding cassette transporters. *Mol. Nutr. Food Res.* **2011**, *55*, 979-988.

243. Wang, M.; Qi, H.; Li, J.; Xu, Y.; Zhang, H. Transmembrane transport of steviol glucuronide and its potential interaction with selected drugs and natural compounds. *Food Chem. Toxicol.* **2015**, *86*, 217-224.

244. Wong, C. C.; Botting, N. P.; Orfila, C.; Al-Maharik, N.; Williamson, G. Flavonoid conjugates interact with organic anion transporters (OATs) and attenuate cytotoxicity of adefovir mediated by organic anion transporter 1 (OAT1/SLC22A6). *Biochem. Pharmacol.* **2011**, *81*, 942-949.